PHARMACOKINETICS AND PHARMACODYNAMICS OF NITROGLYCERIN AND ASPIRIN

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TABLE OF CONTENTS

SUMMARY

The pharmacokinetics and pharmacodynamics of nitroglycerin and aspirin have been evaluated with emphasis being placed on the *in vitro* and *in vivo* metabolism of these drugs in various tissues of the body.

Nitroglycerin (GTN) was metabolized by blood components to the dinitro- (GDN) and in turn mononitroglycerin (GMN) metabolites. The rate of GTN and GDN metabolism was concentration dependent. The rate of GTN metabolism was reduced in the presence of GDNs and prevented by iodoacetamide which was used to stabilize GTN in blood samples obtained for pharmacokinetic studies.

Intravenous (IV) bolus doses of GTN administered to sheep evoked rapid and substantial changes in haemodynamic parameters in a dose-dependent manner. The magnitude of the responses was related to the peak GTN femoral arterial (A) or venous (V) plasma concentration. Large differences in peak GTN concentrations and area under the plasma-time curves (AUC) for A and V plasma were observed. The extent of this femoral A-V gradient, and of gradients across the liver and lungs, was further examined at apparent steady state plasma GTN concentrations attained by continuous IV GTN infusions in sheep. These studies demonstrated substantial extra-hepatic metabolism of GTN; the availability of GTN was in the rank order leg < liver < lung. *In vitro* studies showed that GTN was metabolized to GDNs and GMNs by sheep liver, leg muscle, lung, venous and arterial tissue homogenates and that the presence of GDNs reduced the rate of GTN metabolism. Administration of bolus doses of GDNs to sheep receiving GTN infusion reversed

the femoral A-V gradient and reduced the systemic clearance of GTN. GDN and GMN pharmacokinetics were monitored in some sheep receiving GTN infusions.

Extra-hepatic metabolism of aspirin (ASA) was also investigated in the sheep. During continuous IV ASA infusions an A-V gradient in apparent steady state plasma ASA concentrations across the leg and liver was observed but not across the lungs. In vitro studies of the metabolism of ASA by sheep leg muscle, liver and lung tissue homogenates showed that hydrolysis of ASA to salicylic acid (SA) occurred in all three tissues. The presence of SA had no effect on the rate of ASA hydrolysis by any of the tissues. Bolus doses of SA administered during ASA infusions failed to modify the pharmacokinetics or A-V gradients of plasma ASA concentrations except at the largest SA dose when the rate of elimination of ASA was increased. This was due to the displacement, by SA, of ASA from plasma protein binding sites. ASA metabolite pharmacokinetics were monitored in sheep receiving ASA infusions.

Human platelet function was used to evaluate the pharmacodynamics of ASA administered as a single 300 mg dose of a soluble or a slow-release preparation. Despite marked differences in peak plasma ASA concentrations and AUCs, there was similar inhibition of platelet function elicited by the two doses of ASA. Dose ranging studies using the slow-release preparation once daily for 7 days indicated that maximal inhibition of platelet function occurred using doses of 100-200 mg ASA daily.

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This thesis contains no material which has been accepted for the award of any other degree in any University or College and to the best of my knowledge and belief the thesis contains no material previously published or written by another person except when due reference is made in the text of the thesis.

Signed,

Paul A. Cossum

LIST OF ABBREVIATIONS

AA	arachidonic acid
ASA	acetylsalicylic acid, aspirin
AUC	area under the plasma concentration-time curve
GA	gentisic acid
GC	gas chromatograph(y)
GC-MS	gas chromatography - mass spectrometry
GDN	glyceryl dinitrate
GMN	glyceryl mononitrate
GTN	glyceryl trinitrate, nitroglycerin
HPLC	high performance liquid chromatography
ISDN	isosorbide dinitrate
ISMN	isosorbide mononitrate
IV	intravenous
MABP	mean arterial blood pressure
MDA	malondialdehyde
MLVP	mean left ventricular pressure
NaSA	sodium salicylate
NEM	N-ethylmaleimide
ng	nanogram
6-oxo-$PGF_{1\alpha}$	6-oxo-prostaglandin $F_{1\alpha}$
pg	picogram
PGI_2	prostaglandin I_2, prostacyclin
SA	salicylic acid
SU	salicyluric acid
TBA	thiobarbituric acid
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2
µg	microgram

CHAPTER 1

INTRODUCTION

The discipline of therapeutics has made many advances in recent years. New substances are continually being evaluated for their clinical potential. However, in only a small number of cases is that potential ever converted and the new drug eventually marketed. Often a drug has only one therapeutic indication and so its clinical use is limited. Two drugs which fit neither of these categories are GTN and ASA. Both drugs have been in clinical use since the 19th century for numerous clinical indications.

GTN was first synthesized by Sobrero in Italy in 1846. This was achieved by mixing glycerin with cold, concentrated sulphuric and nitric acids. It was not long before others had repeated the synthesis and found, as Sobrero had, that a small amount of the product placed on the tongue evoked throbbing at the temples and a severe headache (Krantz, 1975). Two Americans, Constantin Hering and Morris Davis, both associated with a Philadelphia medical school, distributed GTN to physicians who were requested to record the effects of GTN when given to their patients. Hering and Davis were keen to compare the effects of GTN and amyl nitrite which had earlier been shown by T. Lauder Brunton in England to alleviate the symptoms of angina pectoris despite causing severe headaches and an increased pulse rate. It is not clear what the results of that study were (Krantz, 1975) and it was not until 1879 that Edward Murrell, an English physician,

conducted similar experiments to find that the efficacy of GTN was similar to that of amyl nitrite in the treatment of angina pectoris.

At about the same time as the therapeutic benefits of GTN were being realized a Swedish engineer, Alfred Nobel, was devising ways to exploit the other major property of GTN, that of its explosiveness. By mixing GTN with charcoal or diatomaceous earth, he made possible the relatively safe handling of GTN as dynamite (Urbanski, 1965). The fortune amassed by Nobel as a result of patents covering dynamite makes possible the current award of Nobel Prizes.

The history of ASA is intricately related to that of other salicylates, all of which have the 2-hydroxy benzoate radical. Salicylates occur naturally in the bark, leaves and fruit of many plants and trees. It is the bark of species of Salix and Populus, however, which have been most widely used as a source of these compounds. Salicylates have been used for many hundreds of years as analgesics and anti-pyretics. Full reviews of the history of salicylates have been presented by Gross and Greenberg (1948) and most recently by Rainsford (1984).

ASA was first synthesized in 1853 by a French chemist, Charles von Gerhardt, by the treatment of sodium salicylate with acetyl chloride. However, credit for the therapeutic use of ASA goes to Felix Hoffman, a chemist at Friedrich Bayer and Company in Germany, who was searching for a better tolerated salicylate than the sodium salt of SA for his father suffering rheumatoid arthritis. Heinrich Dreser, director of pharmacological research at Bayer, believed ASA to be superior to sodium salicylate in terms of less gastrointestinal irritation and began marketing ASA in 1899 (Gross and Greenberg, 1948; Rainsford, 1984). However, as Rainsford (1984) points out, this

promise was not substantiated and in fact ASA is more irritant to the stomach than either sodium salicylate or SA. Nevertheless, the analgesic, anti-inflammatory and anti-pyretic properties of ASA have made it a very valuable drug.

Although GTN and ASA are two of the oldest drugs still in current therapeutics, uses for both drugs have continued to increase. In recent years the ability of GTN to decrease the size and/or severity of acute myocardial infarction has been investigated (Epstein et al., 1975; Flaherty et al., 1975, 1976). Moreover new transdermal formations of GTN have been investigated as alternatives to oral or sublingual dosing of GTN in angina pectoris and in peripheral occlusive vascular disease (Black, 1982; Demma and Wilson, 1983). GTN sales ranked 152 out of the top 200 prescription drugs in the U.S.A. for 1982 (U.S. Pharmaceutical Manufacturers' Association, personal communication). Almost 32 million prescriptions were written for GTN in 1983 in the U.S.A. alone, an increase of about 10 million prescriptions on 1982 (Pharmaceutical Data Services, Arizona, personal communication).

Interest in ASA has grown based on the work of Vane and coworkers who showed that ASA inhibits prostaglandin synthesis generally, and by Smith and Willis (1971) who showed that aspirin inhibits platelet prostaglandin synthesis. Elwood et al. (1974) reported results suggesting that ASA may be beneficial in reducing the mortality of myocardial re-infarction and it is believed that this is related to ASA's anti-platelet effects. A tally of prescriptions for ASA is impossible since even with a prescription, pharmacists (in most parts of the world) sell it to patients as an OTC product and do not record the prescription. Nevertheless, production of ASA in the U.S.A. alone was

estimated as 97 million kilograms in 1980, a large increase from the 1965 figure of 13 million kilograms (Rainsford, 1984).

Despite the enormous use and therapeutic benefits of these two compounds the dosing requirements for, and plasma drug concentrations associated with certain aspects of their therapeutic use remain to be clarified. This may have been due to technical difficulties with regard to the analysis of plasma drug concentrations or to the relative novelty of the therapeutic indication. While empiric dosing of the two drugs for certain indications is being performed without apparent disadvantage to patients, studies to determine some dose-response and plasma drug concentration-response relationships are warranted. Moreover, a fuller understanding of certain aspects of the metabolism of these two drugs is needed as a basis for the development of rational dosing regimens for various indications. This thesis reports the results of studies to examine some of these aspects in sheep and man.

CHAPTER 2

CLINICAL PHARMACOLOGY OF NITROGLYCERIN AND ASPIRIN

2.1. SITE AND MODE OF ACTION

2.1.1. Nitroglycerin

The principal pharmacological action of GTN is to relax vascular smooth muscle. In the peripheral circulation the major effect is to cause venodilatation which results in venous pooling and a reduction in venous return (preload), left ventricular filling pressure, and pulmonary artery and capillary pressures. Myocardial oxygen consumption or demand may be decreased by an associated reduction in systemic blood pressure. Reduction in afterload, although to a lesser extent than preload, is achieved by dilatation of arteriolar resistance vessels of the peripheral circulation. In the coronary arterial circulation GTN dilates both large conductance and small resistance vessels (Parratt, 1974; 1979; Williams, 1981; Flaherty, 1982; Elkayam and Aronow, 1982). A feature of organic nitrate drug therapy can be the development of tolerance towards the drugs with time (Zelis and Mason, 1969; Needleman and Johnson, 1973). Other investigators (Abrams, 1980; Franciosa and Cohn, 1980) have failed to observe nitrate tolerance to be a clinical problem in chronic therapy.

Many ideas have been put forward to explain the way in which GTN relaxes vascular smooth muscle. These range from a specific organic

nitrate receptor to a direct effect on Ca^{2+} movements (Bennett and Marks, 1984).

Needleman and Johnson (1973) proposed the existence of an organic nitrate receptor based on their results obtained with aortic strips from GTN-tolerant rats and rabbit aortic trips made tolerant to GTN in vitro (Figure 2.1.). Treatment with the sulphydryl (SH) reducing agent dithiothreitol reversed the tolerance. Needleman and Johnson (1973) suggested that GTN could react with a SH group of the receptor and, as a consequence of this reaction, smooth muscle would relax, an effect accompanied by partial denitration of GTN and oxidation of the SH group to the disulphide form. Tolerance, according to this theory, was associated with the SH group of the receptor being in the disulphide form, therefore displaying less affinity for GTN. Various other substances which react with SH groups were tested for their ability to reduce GTN-induced relaxation by Moffat et al. (1982). However, because of variations in the lipophilicity of the compounds used, and therefore their ability to penetrate tissue to get to the "biophase", those workers could not produce conclusive evidence to either refute or support the theory of Needleman and Johnson (1973).

Evidence that increased levels of cGMP may mediate vasodilatation has been provided by Diamond and Blisard (1975) and Kobayashi et al. (1980) who found that cGMP levels increased prior to vasodilatation. The presumed increase in guanylate cylase activity has been suggested by Ignarro et al. (1981) to be caused by S-nitrosothiols which form when nitric oxide (derived from GTN metabolism) combined with SH-containing compounds (Figure 2.2.). This theory is dependent on the fact that GTN is denitrated to release NO_2^- which is then converted to nitric oxide. Therefore metabolites of GTN must be formed

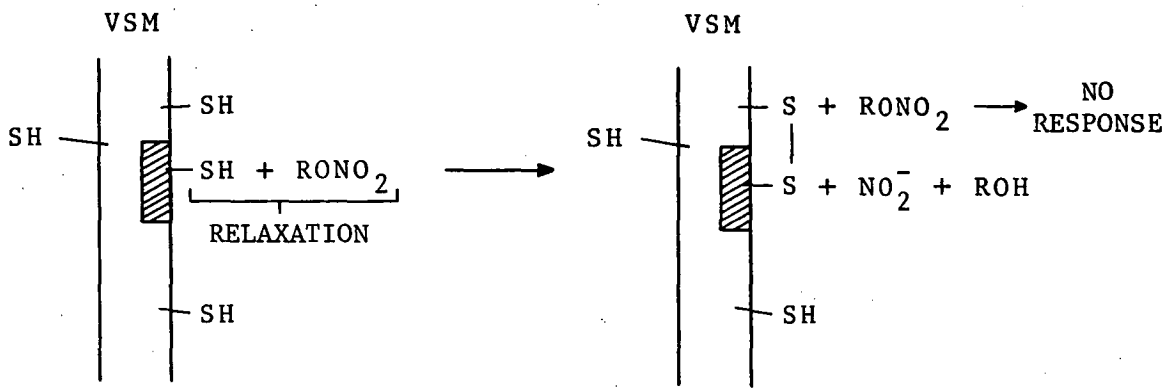


Figure 2.1 Schematic representation of the reaction of GTN ($RONO_2$) with sulphydryl groups (SH) of the vascular smooth muscle receptor as hypothesized by Needleman and Johnson (1973). VSM = vascular smooth muscle, ROH = denitrated metabolite.

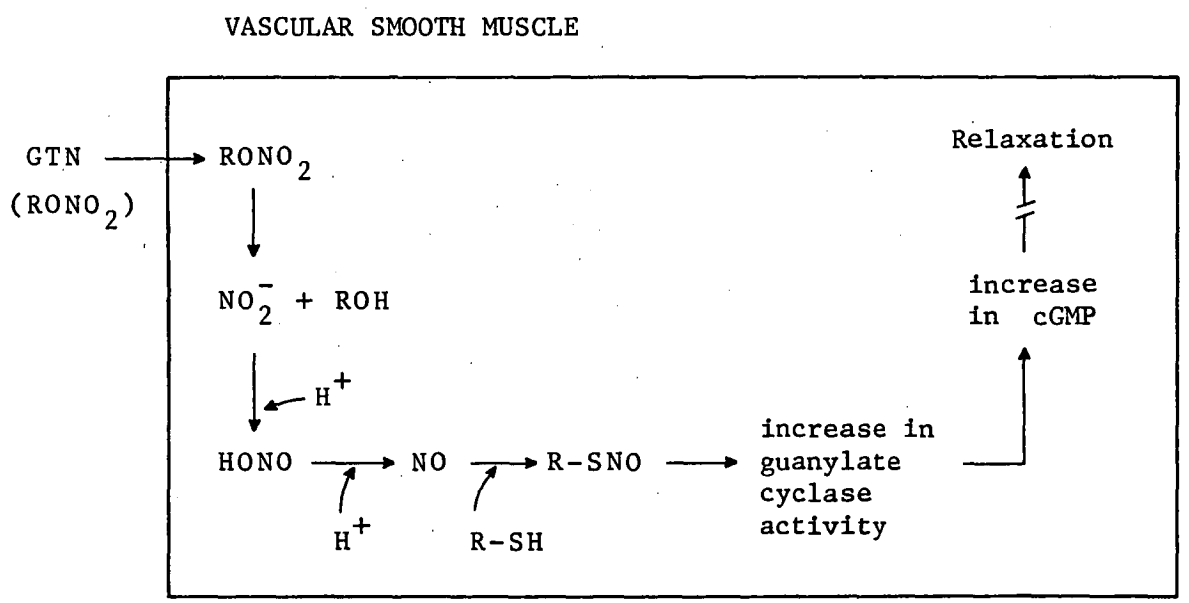


Figure 2.2 Mechanism of GTN-induced relaxation hypothesized by Ignarro et al. (1981). ROH = denitrated metabolite, R-SNO = S-nitrosothiol and R-SH = thiol.

concomitantly. However, no GTN metabolites were detected in the incubation medium during GTN-induced relaxation of isolated canine blood vessels (Armstrong et al., 1980b).

Speculation that prostaglandins (specifically PGI_2) may mediate GTN-induced relaxation of vascular smooth muscle arose from studies which demonstrated that GTN enhanced PGI_2 release (as measured by its stable degradation product, 6-oxo-$PGF_{1\alpha}$) from cultured endothelial cells (Levin et al., 1981), isolated bovine coronary artery (Schror et al., 1981), human saphenous vein (Mehta et al., 1983) and rat aorta (Anderson et al., 1980). All of those studies relied on in vitro pharmacological inhibition of the cyclooxygenase enzyme to identify the relevance of prostaglandin formation to the action of nitroglycerin. Other studies have provided evidence to suggest that PGI_2 is not involved in the mediation of GTN-induced vascular smooth muscle relaxation (Furchgott et al., 1981; Fitzgerald et al., 1984).

Relaxation of muscle is ultimately dependent on intracellular Ca^{2+} concentrations. GTN could induce relaxation by:

(i) altering the Ca^{2+} content of intracellular storage sites, or

(ii) altering the flux of Ca^{2+} across the plasma membrane.

Little work has been done on these aspects of GTN-induced relaxation. However some support for the second mechanism outlined above comes from the work of Ginsburg et al. (1981) who demonstrated increased Ca^{2+} efflux from human coronary arteries after treatment with GTN.

2.1.2. Aspirin

It has been suggested that ASA exerts its therapeutic effects by uncoupling oxidative phosphorylation (Adams and Cobb, 1958), inhibition of histamine or serotonin or blockade of tissue response to those

mediators (Spector and Willoughby, 1968), inhibition of kinin generation and action (Collier, 1969) or interactions with various enzyme systems (Smith and Dawkins, 1971) most particularly the enzymes involved with prostaglandin biosynthesis (Vane, 1971). More recently, research on this aspect of the mode of action of ASA has included inhibition of not only prostaglandins but also thromboxanes and leukotrienes (collectively called eicosanoids). Recent reviews on these topics have been produced by Rainsford (1984) and Bakhle (1983). It is this last effect of ASA which is currently accepted as its mode of action, notwithstanding that the inhibition of eicosanoid biosynthesis may be intricately linked with any or all of the other effects mentioned above.

Arachidonic acid is the specific precursor for the synthesis of eicosanoids. It is released from phospholipid stores in the cell membrane by the action of phospholipases. AA is then available to be metabolized to prostaglandins and thromboxanes or to leukotrienes (Figure 2.3.). The pathway for the synthesis of prostaglandins and thromboxanes proceeds through the combination of the cyclooxygenase/peroxidase catalyzed reactions of the prostaglandin endoperoxides. The resulting endoperoxide, PGG_2, is converted to PGH_2 which is available for conversion to a variety of substances (Samuelson and Hamberg, 1974), (Figure 2.3.). AA is also metabolized to a series of hydroxy and hydroperoxyacids such as HETE (hydroxyeicosatetraenoic acid) and HPETE (hydroperoxyeicosatetraenoic acid) by the lipoxygenases. Comprehensive reviews on the pharmacological roles of prostaglandins and other eicosanoids have been presented by Flower (1974) and Moncada and Vane (1979).

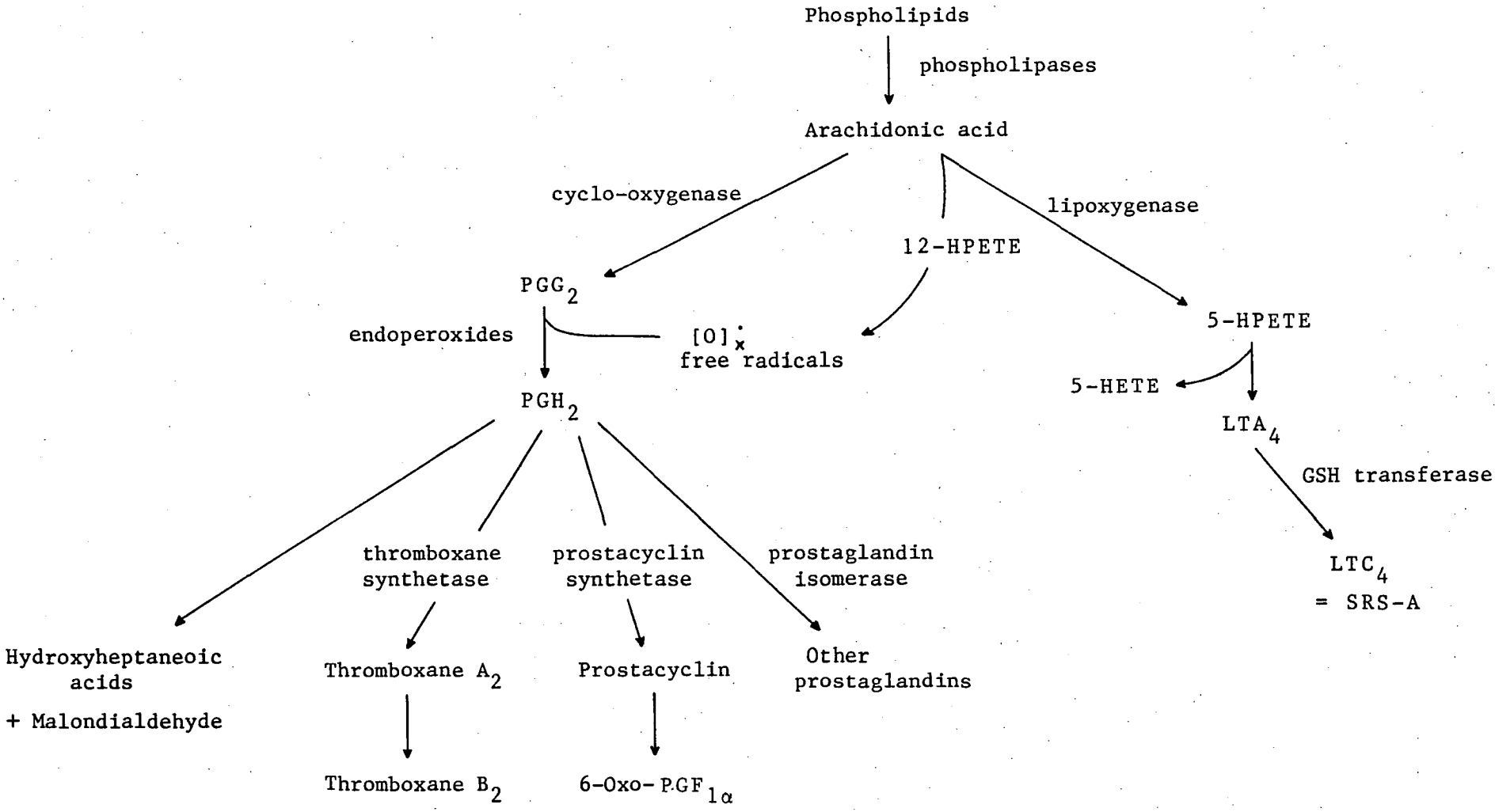


Figure 2.3 Schematic representation of pathways of eicosanoid biosynthesis (Adapted from Rainsford,(1984))

ASA irreversibly inhibits the cyclooxygenase enzyme (Roth et al., 1975) thereby preventing the formation of the endoperoxides, prostaglandins, thromboxanes and MDA (Figure 2.3.). It is this inhibition which is believed responsible for the therapeutic effects of ASA. However the inhibition of cyclooxygenase by ASA may be prevented or reduced by SA (Vargaftig, 1978; DeJana et al., 1981).

2.2 CLINICAL USES

2.2.1 Nitroglycerin

Angina pectoris due to coronary artery disease is generally considered to be chest pain due to myocardial ischaemia which occurs because the myocardial oxygen demand exceeds the myocardial oxygen supply. Up until recent times (the last 20 years) this view emphasized that myocardial demand for oxygen exceeded the flow capability of narrowed coronary vessels. A review of this topic by Aronow (1975) demonstrates that the over-riding line of thinking was that angina was due to atherosclerotic disease of coronary vessels. More recently, angina has been described as a vasospastic disorder of the arteries of the heart, and indeed coronary vasospasm has now been firmly established as the mechanism of ischaemia in Prinzmetal's variant angina. Nairn and Friedlander (1983) point out that spasm may be one extreme in the concept of dynamic coronary artery obstruction and that more modest changes in the tone of larger epicardial coronary vessels can vary the degree of obstruction. Endogenous substances such as serotonin, endoperoxides and some prostaglandins and thromboxanes produced by the coronary arteries and platelets are potent vasoconstrictors and may have a large role to play in the genesis of myocardial ischaemia

(Nairn and Friedlander, 1983; Hamberg et al., 1975). GTN (and other organic nitrate drugs) reduces coronary vasospasm in normal and atherosclerotic vessels by virtue of its smooth muscle relaxant properties.

Treatment of acute left ventricular and chronic heart failure is achieved by administering GTN to cause a significant reduction in afterload by reducing the compensatory peripheral resistance of heart failure and thereby improving cardiac output. GTN is also used to induce controlled hypotension during diverse surgical procedures (Franciosa, 1980; Franciosa and and Cohn, 1980; Hill et al., 1981; Flaherty, 1982).

A more controversial clinical use of GTN is to reduce the size of an acute myocardial infarction. Earlier reports (Epstein et al., 1975; Flaherty et al., 1975; 1976) indicated that IV GTN reduced ST segment elevation and infarct size however these results have been treated cautiously. Elkayam and Aronow (1982) point out that conclusive evidence that GTN can reduce the size of an acute myocardial infarction awaits reports of large scale clinical trials in carefully selected patients using improved techniques of estimating infarct size.

2.2.2. Aspirin

ASA is a widely used drug for the relief of pain of moderate intensity, postoperative pain and cancer pain (Mahler et al., 1976; Ventafridda et al., 1975). Although it appears that ASA does not prevent the formation or release of pain mediators other than prostaglandins, it does remove the nerve sensitizing effect of the prostaglandins thereby raising the pain threshold (Vane, 1978).

Although ASA is used as an anti-pyretic drug the exact mechanism by which it lowers the body temperature is not known (Flower et al., 1980) although prostaglandin synthesis inhibition and/or the augmentation of heat dissipation are believed to be involved (Rainsford, 1984).

ASA and SA are still the most commonly prescribed drugs for the treatment of the inflammation associated with rheumatoid arthritis (Moncada et al., 1980). No one biochemical action of ASA (and other salicylates) is sufficient to account singularly for the therapeutic effects of these drugs as anti-inflammatory agents. Recent reviews (Atkinson and Collier, 1980; Packham, 1982) have centered their attention on the prostaglandin biosynthesis inhibition of salicylates as the major mechanism of action in the treatment of the inflammation of rheumatoid arthritis and other conditions. Atkinson and Collier (1980) regard effects of salicylates on leucocyte migration, lymphocyte functions and superoxide production only in relation to prostaglandin production, a view which does not consider the fact that such functions may be independent of prostaglandins (Rainsford, 1984). Indeed Dawson (1979) was unable to find any correlation between the ability of drugs to inhibit prostaglandin synthesis and their efficacy in in vivo models of inflammation. Bonta and Parnham (1978) showed that the effects of prostaglandins in acute inflammation may not be the same in chronic inflammation such as is associated with rheumatoid arthritis. One example is that capillary permeability appears to become desensitized to prostaglandins during the chronic inflammation phase.

ASA is being used clinically as an anti-thrombotic agent to reduce the incidence of (further) attacks of transient cerebral

ischaemia and hopefully lower the incidence of stroke (Fields et al., 1977; The Canadian Co-operative Study Group, 1978) and to prevent myocardial reinfarction (Elwood and Sweetnam, 1979; Aspirin Myocardial Infarction Study Research Group, 1980). A recent comprehensive review by Hirsh (1982) covers the rationale of using ASA for these indications and its clinical effectiveness.

The rationale for the use of ASA as an anti-thrombotic drug is intricately related to ASA's ability to inhibit platelet function. The contribution of platelets in haemostasis and thrombosis is related to their ability to adhere to non-endothelialized surfaces, to undergo a platelet release reaction, to aggregate and to potentiate blood coagulation. Briefly, platelets do not adhere to normal endothelium but adhere to collagen which is exposed when there is a break in the endothelial lining of blood vessels (Cazenave et al., 1979). Once adherence has occurred, the platelets change shape and release the contents of storage granules containing numerous substances including coagulation factors (Kaplan et al., 1979). Other granules secrete adenosine diphosphate (ADP), serotonin and calcium. ADP induces aggregation of platelets and serotonin induces vasoconstriction proximal to the release site and helps reduce blood flow so that a platelet aggregate can be formed and build (Vreeken, 1982). Other mediators of platelet aggregation are thrombin and TXA_2.

Thromboxane is produced from AA, a pathway that involves cyclooxygenase and thromboxane synthetase (Figure 2.3.). TXA_2 is a potent vasoconstrictor as well as an inducer of platelet aggregation. Another eicosanoid, PGI_2, is synthesized, via cyclooxygenase and PGI_2 synthetase, from AA in the endothelium of blood vessels (Moncada et

al., 1976). It has been shown to be a potent anti-platelet aggregating factor and vasodilator (Moncada and Vane, 1978). Since ASA irreversibly acetylates cyclooxygenase (Roth et al., 1975) it would be anticipated that synthesis of TXA_2 and PGI_2 would be equally affected by ASA. However there is evidence that vascular cyclooxygenase is less sensitive to ASA than platelet cyclooxygenase (Burch et al., 1978a; Basista et al., 1978). Moreover, inactivated vascular cyclooxygenase may be replaced relatively rapidly by the enzyme synthesized de novo in the endothelium (Jaffe and Weksler, 1979), whereas platelets do not possess appreciable protein synthesis capabilities and so the complete restoration of circulating platelet cyclooxygenase activity depends on replacement of the total affected platelet population which takes 8-14 days (Burch et al., 1978b; Stuart et al., 1975). This apparent difference in cyclooxygenase sensitivity to ASA has led to the development of the PGI_2/TXA_2 balance hypothesis which postulates that the ratio of these two substances determines platelet aggregability and thrombosis tendency (Moncada and Vane, 1978).

Studies to investigate the presumed benefits of ASA therapy to reduce the risk of thrombosis have as their basis a desire to choose the appropriate dosage of ASA which maximally inhibits platelet TXA_2 and thus a degree of aggregation, while leaving intact the PGI_2 synthetic pathways of the endothelium of the vessel wall. Indeed, platelet TXA_2 production could be almost completely blocked by small daily doses of ASA (Masotti et al., 1979; Hanley et al., 1981). An accurate assessment of the role of ASA in modifying the PGI_2/TXA_2 balance in favour of PGI_2 has not been forthcoming due to the lack of suitably accurate and sensitive assays of PGI_2 production in vivo.

To overcome this problem most assays of PGI_2 have involved quantitating concentrations of 6-oxo-$PGF_{1\alpha}$, a stable metabolite of PGI_2 (Salmon, 1978). Results obtained using radioimmunoassay (Mitchell, 1978) lack the specificity necessary for measurements in plasma, while those based on GC-MS operate close to the limit of sensitivity (Hensby et al., 1979). Blair et al (1982) developed a GC-MS assay using capillary column GC and negative ion chemical ionization MS which offered greater specificity and sensitivity than their previous GC-MS assay and which was used to show that PGI_2 is probably a local hormone rather than a circulating hormone in man.

As well as TXA_2 (or its more stable metabolite, TXB_2) production being used as an index of platelet function, the measurement of platelet aggregators to induce <u>in vivo</u> aggregation can be used to assess platelet function (Born, 1962). Typical aggregating inducers used have been collagen, adrenalin, ADP, thrombin and AA (Born, 1962; Siess et al., 1981). Moreover platelet cyclooxygenase activity can be assessed by the ability of platelets to produce MDA, an end-product from AA metabolism (Figure 2.3.) (Flower et al., 1973).

CHAPTER 3

ANALYSIS AND PHARMACOKINETICS OF NITROGLYCERIN AND ASPIRIN

3.1. METHODS OF ANALYSIS

3.1.1. Gas Chromatography

The mainstay of GC assays of GTN plasma concentrations has been that developed by Rosseel and Bogaert (1973). This assay involved extraction of GTN from plasma using ethyl acetate, and, after injection onto a GC column, quantitation of GTN by means of a ^{63}Ni electron capture detector. Modifications of this method have mostly entailed using hexane instead of ethyl acetate as the extracting solvent because a cleaner chromatogram is obtained with hexane (Yap et al., 1978; Armstrong et al., 1979). However only a limited amount of work involving plasma GTN concentrations has appeared in the literature despite the above methods appearing some years ago. This may be due to the difficulties associated with GTN having a large vapour pressure (Urbanski, 1965), and adsorption of small amounts of GTN to diatomaceous earth GC column packings (Urbanski, 1965), or glass (Yap et al., 1978) or plastic (Cossum et al., 1978) containers. A limitation of GC assays of organic nitrates is that GDN metabolites are only slightly soluble in hexane and so are not extracted from plasma in quantitative amounts, unlike GTN (Yap et al., 1978). Moreover a suitable GC packing to separate the GDN isomers and GTN is not available (at the

start of these studies). Therefore pharmacokinetic analysis of GDN metabolites has not been possible using current GC techniques.

Rowland and Riegelman (1967) developed a GC method to determine plasma ASA concentrations using the trimethylsilyl derivative and flame ionization detector after extraction of ASA from plasma by diethyl ether. Later methods have been described which determine ASA and SA plasma concentrations simultaneously (Thomas et al., 1973; Rance et al., 1975). Complications of GC analysis of plasma salicylate concentrations include multiple product formation with the derivatizing agent (Rowland and Riegelman, 1967) and partial hydrolysis of aspirin during derivatization (Ali, 1975). These assays are also time consuming with extraction and derivatization generally taking over 90 min.

3.1.2. High Performance Liquid Chromatography

Although HPLC assays of GTN and its metabolites have been described using UV detectors (Baaske et al., 1979; Crouthamel and Dorsch, 1979), they are only suitable for analyzing large drug concentrations (as in pharmaceutical preparations) owing to the limited absorptive properties of GTN and its metabolites. Nevertheless, the HPLC column is a useful tool for the separation of GTN from its GDN and GMN metabolites, and the isomeric GDN metabolites from each other and the GMN metabolites from each other. This property has been used by Spanggord and Keck (1980) to analyze blood GTN and metabolite concentrations following oral administration of GTN to dogs. The method of detection was the thermal energy analyzer.

Several HPLC methods for the determination of salicylates in body fluids have been described. Some measure ASA and SA (Caterson et al.,

1978), others measure ASA, SA and salicyluric acid (SU) in plasma but with a time-consuming extraction process (Peng et al., 1978), or measure SA, SU and GA, but not ASA, in plasma (Cham et al., 1979). Rumble et al. (1981) produced a method which offered simultaneous determination of ASA, SA, SU and GA plasma concentrations without solvent extraction.

3.2. PHARMACOKINETICS OF NITROGLYCERIN

The pharmacokinetics of GTN has traditionally been associated with a short half-life of elimination, reported to arise from a significant or even complete hepatic first-pass effect. These beliefs have arisen from the early work of Needleman's and DiCarlo's groups using different animal species (Needleman and Hunter, 1965; Needleman, 1973; DiCarlo et al., 1968).

3.2.1 Absorption

The absorption of GTN was rapid after oral administration of ^{14}C-GTN to rats (DiCarlo et al., 1968), however this route of administration has not been used widely since it is believed that the liver is responsible for the complete inactivation of GTN during its first-pass through the liver (Needleman, 1973) (see below). For this reason the sublingual route of GTN dosing has been the route of choice for rapid relief of the pain of angina pectoris, suggesting that sublingual absorption of GTN is rapid. Indeed, peak plasma concentrations of GTN given sublingually appear within 2 to 4 min (Bogaert and Rosseel, 1972a; Blumenthal et al., 1977; Armstrong et al., 1979). Following administration of a 600 µg GTN tablet, peak venous plasma GTN concentrations were 2.3 ng/ml (Armstrong et al., 1979).

Percutaneous dosing of GTN is now performed routinely using ointments and, most recently, transdermal patches (an inert matrix releasing GTN at a constant rate). These formulations are used clinically to prolong the absorption of GTN and to avoid substantial degradation of GTN due to a liver first-pass effect. Recent reviews on the clinical uses and biopharmaceutics of percutaneous GTN formulations have been provided by Black (1982) and Elkayam and Aronow (1982).

Intravenous dosing of GTN is a common route of administration in patients following acute myocardial infarction with or without heart failure (Hill et al., 1981; Flaherty, 1982). An approximate linear relationship between infusion rate and arterial plasma GTN concentrations has been reported by Armstrong et al. (1980a), however this report awaits verification using suitable IV administration systems. Cossum et al. (1978) showed that the GTN in IV solutions was lost to the polyvinyl chloride of IV administration sets in simulated infusions. GTN concentrations in the effluent were a function of the flow rate of GTN solution through the polyvinyl chloride tubing of the sets, the faster flow rates being associated with the smaller losses of GTN (Figure 3.1). It is not clear whether Armstrong et al. (1980a) used polyvinyl chloride administration sets or polyethylene tubing which was shown by Cossum et al. (1978) to minimize the loss of GTN and later by Cossum and Roberts (1981) to prevent the loss of diazepam, chlormethiazole and isosorbide dinitrate which occurred when these drugs were infused through polyvinyl chloride administration sets.

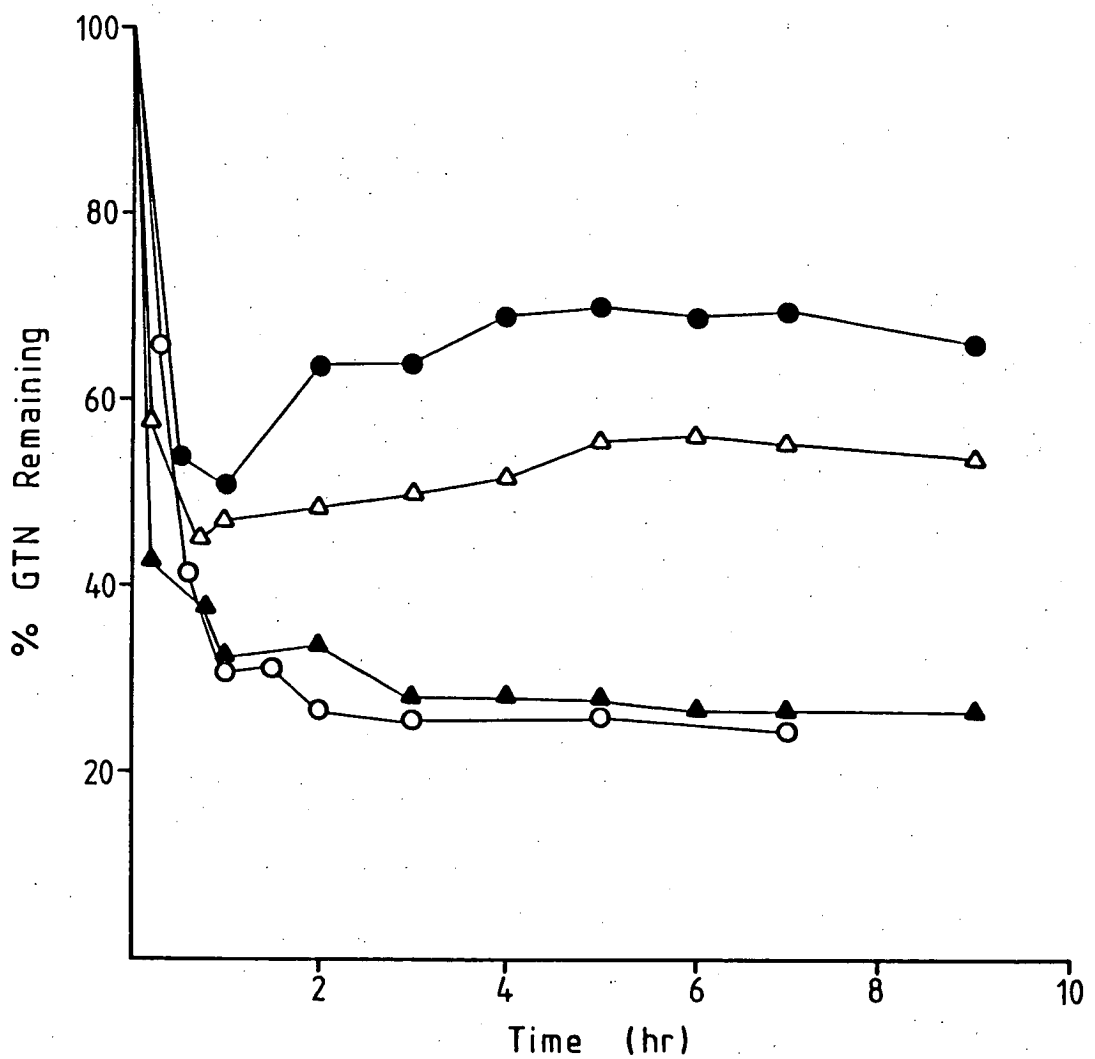


Figure 3.1 Effect of flow rate on the percentage of nitroglycerin (GTN) remaining after passage through plastic infusion sets.
(●) 0.91 ml/min; (△) 0.30 ml/min; (▲) 0.17 ml/min; (○) 0.07 ml/min

3.2.2. Distribution

It has been estimated that only about 1% of the total body load of GTN is in the blood (McNiff et al., 1981). This suggests that tissue distribution of the drug is extensive. However pharmacokinetic calculations of the volume of distribution (V_d) of GTN reported in the literature have been widely varying. For example, Armstrong et al. (1980a) reported a V_d of about 10 litres, but McNiff et al. (1981) reported a V_d of 210 litres. A major difference in the way the two studies were conducted was that Armstrong et al. (1980a) sampled arterial blood from their patients, but McNiff et al. (1981) sampled venous blood from their volunteers. These data suggest that pharmacokinetic data may be dependent on the site of blood sampling.

No reports are available on the degree of binding of GTN or its metabolites to human plasma proteins. However, DiCarlo and Melgar (1969) studied the binding of GTN to rat plasma. They found that about 60% of the GTN was bound to rat plasma proteins. During the course of the binding experiments the GTN was metabolized to 1,3-GDN and 1,2-GDN. The 1,2-GDN was about 60% bound to plasma proteins while 33% of the 1,3-GDN was bound. DiCarlo and Melgar (1969) used a GTN concentration of about 1 mg/ml plasma in these studies, a concentration which is many times greater than therapeutic levels.

3.2.3. Metabolism and Elimination

GTN is metabolized to the pharmacologically less active GDNs, probably by a glutathione-dependent organic nitrate reductase (Needleman and Hunter, 1965), which may be one enzyme of the glutathione transferase group (Habig et al., 1975). The liver is known to

be capable of substantial in vitro GTN metabolism (Needleman and Harkey, 1971). Other sites where glutathione transferase activity and GTN metabolism have been shown to occur are the kidney (Maier et al., 1980) and erythrocytes (Marcus et al., 1978; Armstrong et al, 1980c) although the enzyme isolated from human erythrocytes is distinct from that isolated from human liver. Figure 3.2. shows the patterns of GTN metabolism following perfusion of ^{14}C-GTN solutions through isolated rat livers (Needleman and Harkey, 1971). A comparison of the maximum velocities of the enzymatic transformation of GTN and its metabolites indicated that GTN was metabolized more than 20 times faster than the GDNs, which were in turn metabolized more rapidly than the GMNs by liver organic nitrate reductase (Needleman and Hunter, 1965). Needleman and Krantz (1965) found that the GMNs appeared as the primary urinary metabolites after GTN administration to rats. No GTN was detected in the urine. A small percentage of GTN must be degraded to glycerol since DiCarlo et al. (1969) reported that about 1% of a ^{14}C-GTN dose was recovered from the liver as lipid, glycogen, protein, RNA and DNA.

The rapid half-life of elimination of 1 to 2.8 min for GTN from blood following administration of GTN to intact rats (Johnson et al., 1972) or humans (Rosseel and Bogaert, 1973; Armstrong et al, 1980a; McNiff et al., 1981) is believed to be due to rapid hepatic elimination of GTN. Although erythrocytes also metabolize GTN in vitro (Armstrong et al., 1980c), it has been calculated that this metabolism is responsible for less than 3% of the total body clearance of GTN (McNiff et al., 1981). Clearance values reported by investigators sampling venous blood after GTN administration (Wei and Reid, 1979; Armstrong et al., 1979; McNiff et al., 1981) are in the range 29 to

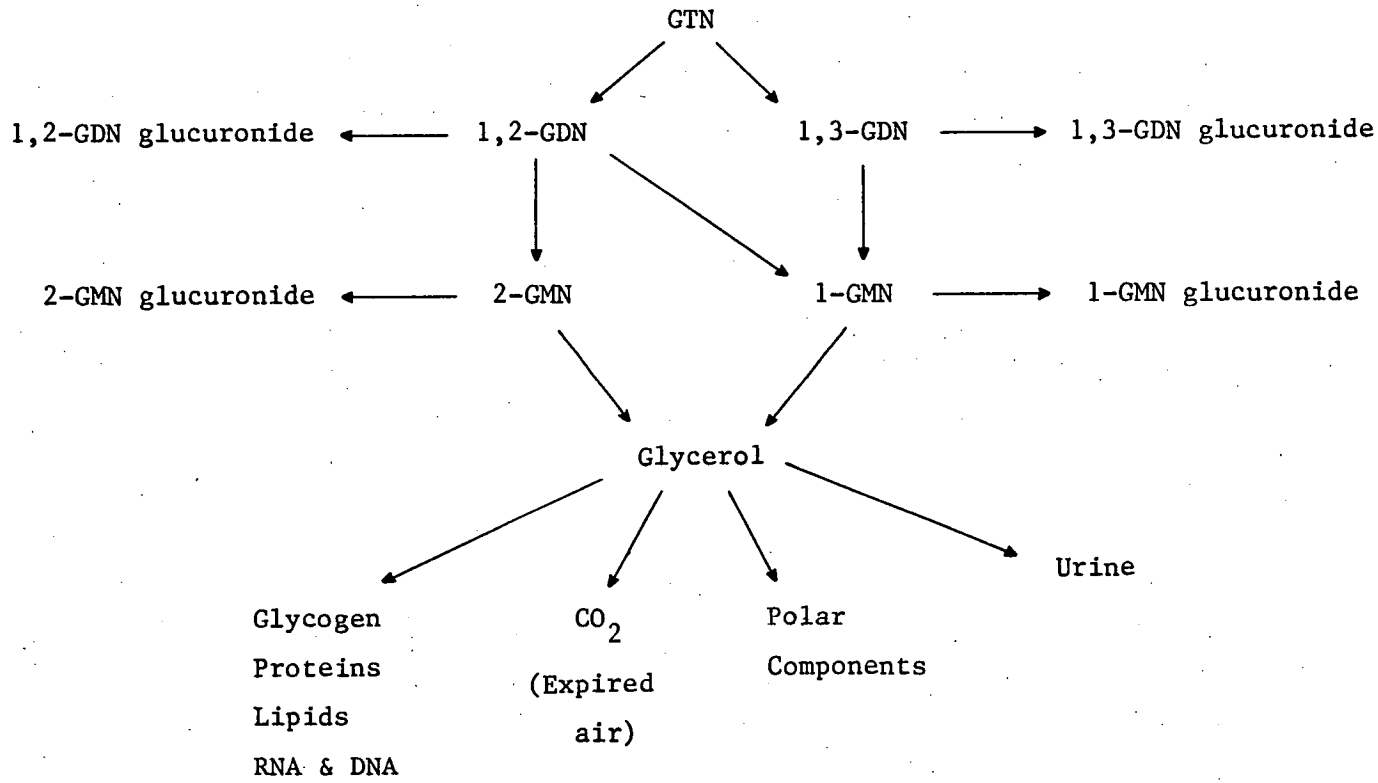


Figure 3.2 Proposed pathway for the metabolism of GTN in rats. (Adapted from Needleman and Harkey, 1971)

82.3 l/min, values which are at least several times the value of normal human cardiac output. Other workers using arterial blood samples have reported GTN clearance values of 4.4 to 13.8 l/min (Idzu et al., 1981; Armstrong et al., 1980a), which approximates normal cardiac output. Since both arterial and venous clearance of GTN is greater than hepatic blood flow, it appears that extra-hepatic metabolism of GTN could play a major role in the clearance of GTN. Moreover, the large difference in arterial and venous GTN clearance as reported by the various groups mentioned above indicates that GTN degradation could occur in the capillary beds and/or muscle tissue and/or arterial/venous tissue. The lungs are potentially another site of GTN metabolism given their role in other xenobiotic metabolism (Mehendale et al., 1981) and the fact that they receive the entire cardiac output.

Studies on another organic nitrate drug, ISDN, may have provided some important avenues of study of the metabolism of GTN. Fung et al. (1981) reported that after chronic oral dosing, plasma ISDN concentrations were higher than would be expected from single oral dose kinetics. ISDN (like GTN) is probably metabolized by an organic nitrate reductase (Needleman and Hunter, 1965) to the 5- and 2-ISMNs (Dietz, 1967). Both metabolites are eliminated more slowly than ISDN (Abshagen and Sporl-Radun, 1981) and this suggests that the increased plasma ISDN concentrations seen after chronic ISDN dosing may be due to an interaction of metabolites and parent compound. If the same were true for GTN and its metabolites then the phenomenon occurring with both organic nitrate drugs may provide information to elucidate the often observed occurrence of tolerance and cross-tolerance to the cardiovascular effects of the drugs in patients on long-term organic

nitrate therapy. The relevance of such pharmacokinetic study is evident when it is considered that two of the current hypotheses put forward to explain organic nitrate drug action on vascular smooth muscle, have as their basis a denitration step (Needleman and Johnson, 1973; Ignarro et al., 1981).

3.3. PHARMACOKINETICS OF ASPIRIN

3.3.1. Absorption

The rate of absorption of ASA is dependent upon many factors; the pH of the gastrointestinal tract, the rate of gastric emptying, the presence of food in the gastrointestinal tract, the volume of fluid ingested with ASA, the formulation of ASA, and others (Levy and Leonards, 1966; Mayersohn, 1977; Orton et al., 1979; Sansom, 1983; Schanker et al., 1958).

In relation to formulation factors, slow-release (enteric coated) ASA tablets were developed to reduce the gastric side effects associated with the very large daily doses needed for the treatment of rheumatoid arthritis. Moreover, the formulation also offered the possibility of prolonged ASA absorption and hence an approximate steady-state salicylate plasma concentration. However the bioavailability of the earlier enteric coated ASA formulations was low and variable (Clark and Lasagna, 1965; Levy and Jusko, 1967). The unpredictable onset of absorption of enteric coated ASA tablets appears to be due mainly to the variability of gastric emptying and so ASA is absorbed from the neutral small intestine in an unpredictable fashion. An enteric coated ASA dosage form which provides more reproducible absorption is that consisting of a large number of

enteric coated granules (Portek et al., 1981; Alpsten et al., 1982). Because of the relatively predictable absorption of ASA from enteric coated granules these formulations are suitable for investigating the association of low but prolonged ASA plasma concentrations with the inhibition of platelet function induced by ASA. Such a study is warranted because in most of the studies investigating optimal ASA dosage regimens for cardiovascular pathology, the soluble dose form has been used with single or chronic dosing (Masotti et al., 1979; Preston et al., 1981; Hanley et al., 1981). Ali et al. (1980) studied the effects of an enteric coated tablet on platelet function.

3.3.2. Distribution

The volume of distribution of ASA in man was reported as 0.1 - 0.2 l/kg (Rowland and Riegelman, 1968). This value was only slightly greater than that found for SA by the same workers. The difference was ascribed to SA being bound to plasma proteins to a greater extent than ASA (Rowland and Riegelman, 1968). Aarons et al. (1980), noting that significant hydrolysis of ASA could occur during *in vitro* protein binding studies of ASA using dialysis, used an ultracentrifugation technique to show that the fraction of ASA bound to albumin was 0.85. They also showed that SA, which is 95% bound, displaced ASA from albumin binding sites and noted that this result may have implications during continuous ASA therapy since SA is the major metabolite of ASA. The binding of SA itself is concentration dependent, a greater unbound fraction of SA being found at the larger SA concentrations (Aarons et al., 1980).

3.3.3. Metabolism and Eliminations

ASA administered orally in aqueous solution is rapidly absorbed but only about 68% of the intact drug reaches the systemic circulation (Rowland et al., 1972). The rest of the dose is hydrolyzed to SA by non-specific esterases in the gut wall and by the liver (Rowland et al., 1972) and blood (Harris and Riegelman, 1967). Moreover, ASA acetylates a variety of proteins in various tissues and salicylate is released from this reaction (Rainsford et al., 1983; Rainsford, 1984). The extent to which other extra-hepatic sites play in the metabolism of ASA is not known.

Rowland et al. (1972) found the half-life of the elimination phase after oral ASA dosing to be in the range 14 to 20.5 min which was slower than the 13 to 19 min observed for the elimination phase after IV ASA dosing (Rowland and Riegelman, 1968). The difference was assumed to be due to the continued absorption of ASA during the elimination phase following oral dosing.

SA, which is formed from the hydrolysis of ASA, undergoes further metabolism; conjugation with glycine to form SU, conjugation with glucuronic acid to form salicyl acyl glucuronide and salicyl phenolic glucuronide, hydroxylation to GA (Figure 3.3). In addition SA is itself renally excreted. The various metabolites of SA are excreted as rapidly as they are formed except in renal failure (Levy, 1978). The kinetics of elimination of SA are characterized by the two quantitatively most important pathways (formation of SU and salicyl phenolic glucuronide) displaying Michaelis-Menten kinetics (Levy et al., 1972). This results in larger SA doses being eliminated at a slower rate than smaller doses (Levy, 1978). As noted in Section 3.3.2. the binding of SA to plasma proteins is concentration dependent also. Therefore an

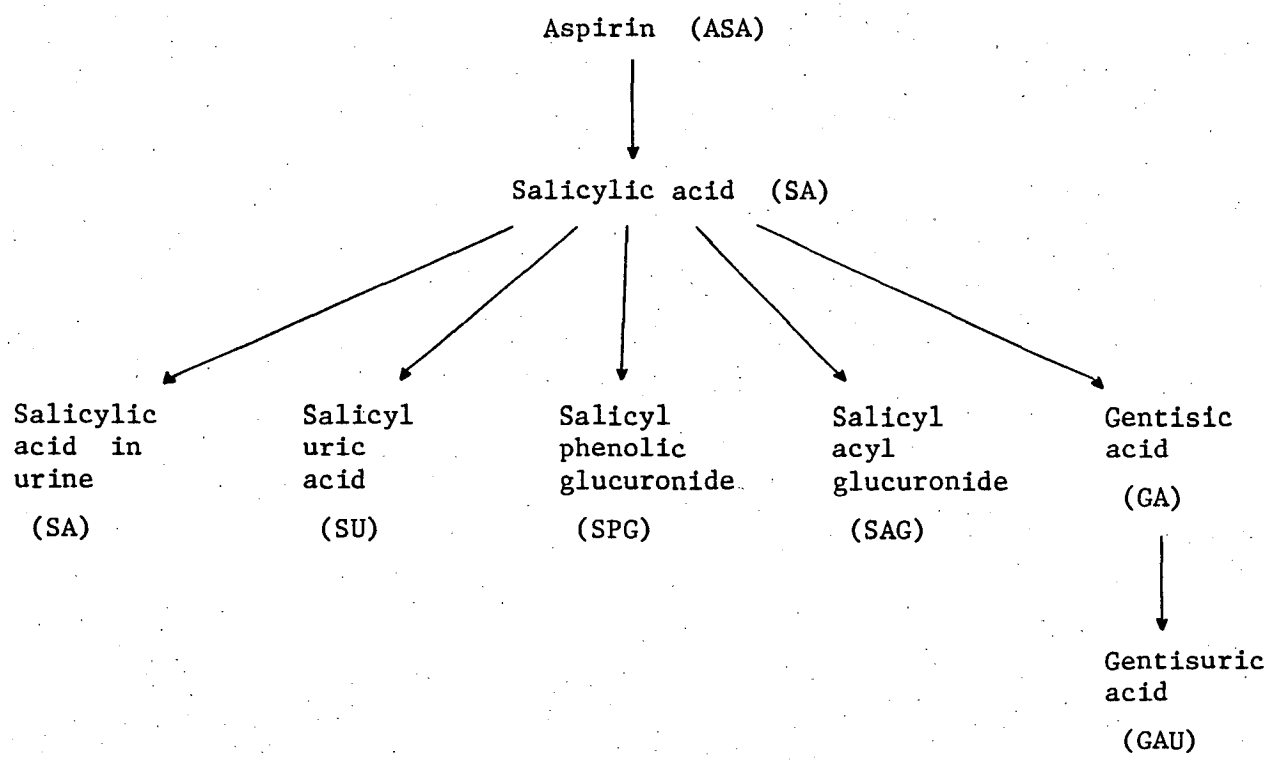


Figure 3.3 Pathway of the metabolism of aspirin.

investigation of the action of SA on ASA plasma protein binding *in vivo* may reveal that continuous ASA dosing at a sufficiently high level could eventually bring about an increase in the elimination of ASA due to the SA metabolite action.

3.4. OBJECTIVES OF THE CURRENT STUDIES

The objectives of the current work were to:

(i) investigate the basis for an apparent difference in GTN concentrations in arterial and venous blood having first overcome stability problems of GTN in blood *in vitro* and in conventional IV administration sets;

(ii) examine the influence of GDNs on the disposition of GTN;

(iii) perform the same studies as above using ASA and to monitor the effects of SA on ASA disposition;

(iv) study the influence of the rate of GTN and ASA administration on their kinetics and on some pharmacological responses evoked by them.

CHAPTER 4

EXPERIMENTAL

4.1 MATERIALS

4.1.1. Chemicals and Drugs

Acetic acid, glacial, analytical grade
Ajax Chemicals, Sydney.

acetonitrile, liquid chromatography grade,
Waters Associates, Melbourne.

(-) adrenalin, 1 mg/ml,
David Bull Laboratories, Victoria.

allyl alcohol, laboratory grade,
May and Baker, Dagenham, United Kingdom.

ammonium hydroxide, analytical grade,
Commonwealth Ammonia Corporation, Melbourne.

arachidonic acid, Grade 1, porcine liver,
Sigma Chemical Co., St. Louis.

benzene, analytical grade,
Ajax Chemicals.

carbon tetrachloride, analytical grade,
Ajax Chemicals.

collagen, equine, 1 mg/ml,
Hormon-Chemie, Brussels.

ethyl acetate, analytical grade,
Ajax Chemicals.

N-ethylmaleimide,
Sigma Chemical Company.

glucose-6-phosphate, monosodium salt,
Boehringer, West Germany.

glucose,6-phosphate dehydrogenase,
Grade 1 suspension, Boehringer.

glutathione, reduced,
Sigma Chemical Company.

glycerol, analytical grade,
Ajax Chemicals.

heparin,

Commonwealth Serum Laboratories.

hexane, pesticide grade,

Fisher.

hydrochloric acid, analytical grade,

May and Baker.

iodoacetamide,

Sigma Chemical Company.

isooctane, analytical grade,

Ajax Chemicals.

isosorbide dinitrate, 40% on lactose,

Schweiz, Sprengstoff-Fabrik.

methanol, liquid chromatography grade,

Waters Associates.

NADPH, tetrasodium salt,

Boehringer

nitric acid, fuming, analytical grade,

Ajax Chemicals.

pentobarbitone sodium (Nembutal) 60 mg/ml,
Abbott Laboratories, Melbourne.

perchloric acid, analytical grade,
British Drug Houses.

phenol, analytical grade,
May and Baker, Australia.

phosphoric acid, analytical grade,
Drug Houses of Australia.

potassium fluoride, laboratory grade,
BDH, Australia.

potassium nitrate, analytical grade,
By-Products and Chemicals, Sydney.

silica gel, GF,
Sigma Chemical Company.

sodium hydrogen citrate, laboratory grade,
British Drug Houses.

sodium carbonate, analytical grade,
Hopkin and Williams Ltd., Essex.

sodium edatate, laboratory grade,

British Drug Houses.

sodium salicylate, laboratory grade,

May & Baker.

sulphuric acid, fuming, analytical grade,

Ajax Chemicals.

thiobarbituric acid,

Ajax Chemicals.

^{3}H - Toluene, certified calibration quality,

New England Nuclear, North Ryde, New South Wales.

trichloroacetic acid, analytical grade,

Ajax Chemicals.

4.1.2 General

All glassware was washed in "Pyroneg", rinsed with distilled water and air dried before soaking overnight in chromic acid. Following thorough rinsing with distilled water the glassware was dried in an oven at 70°C.

Glassware used in experiments involving GTN was silanized by soaking for 2 hr in a 5% solution of trimethyl chlorosilane in toluene. Tubes, flasks, pipettes and syringes were then rinsed in toluene and then oven dried.

Preparation of Drug Solutions

GTN solutions were prepared in glass. Solutions for infusion were prepared immediately prior to an experiment commencing.

GDNs for infusion were prepared by evaporation of the ethyl acetate/hexane of ethanol in which they were dissolved and reconstituted in normal saline immediately prior to their infusion.

ASA solutions for infusion were prepared from commercially available tablets (Aspro Clear). The desired number of tablets were dissolved in a known volume of normal saline 30 min before an experiment commenced. At the time of infusion the aspirin solutions were generally free of bubbles.

NaSA was dissolved in normal saline immediately before its infusion.

ISDN was purified by ether extraction of the powder and recrystallization from an ethanol/water mixture. It was stored as crystals which were dissolved in hexane when needed.

4.2. ANALYTICAL PROCEDURES

4.2.1. Synthesis of Glyceryl Nitrates

Tritiated Nitroglycerin

Prior to the synthesis of ^{3}H-GTN, small scale synthesis of GTN was developed using unlabelled glycerol. In these experiments 1.8 mg glyceryl in 0.1 ml ethanol was transferred to each of 5 tapered glass test tubes and the ethanol evaporated under vacuum to leave about 2.5 µl glycerol which was centrifuged to the bottom of the test tube. Ten µl of a mixture (1:1) of fuming nitric and sulphuric acids

(Urbanski, 1965) was added to the glycerol and each mixture was incubated on ice for 15-90 min. At desired times the reaction in any test tube was terminated by the addition of 5 ml of a 4% aqueous solution of sodium carbonate. The resulting mixed glyceryl nitrates were washed twice with water, which was discarded, and then the small drop of mixed glyceryl nitrates was dissolved in 5 ml ethanol before determining the GTN concentration of the ethanolic solution by HPLC. Calibration curves were constructed using aqueous dilutions of ethanolic GTN commercial solution (Tridil, American Critical Care) which had been analyzed by the USP XX (1979) method. Yields of GTN were calculated as the percentage of theoretical maximal yield from the available mass of glycerol. Incubation times for the maximal yield of GTN were then used in planning the radiochemical synthesis of labelled GTN.

Unlabelled glycerol (1.8 mg in 0.1 ml ethanol) was added to 5 ml of an ethanolic tritium labelled glycerol solution (0.18 mg (1(3)-^{3}H) glycerol, 2.5 Ci/mmol, Amersham Australia). The solutions were incubated using the procedure developed for the synthesis of unlabelled GTN. However for the synthesis of ^{3}H-GTN the incubation time was reduced to give a submaximal GTN yield and an enhanced yield of ^{3}H-labelled GDNs (as they were also required for metabolic studies).

The resulting drop of tritiated glyceryl nitrates was dissolved in 1 ml ethanol and purified by HPLC (Section 4.2.2.). Each compound was obtained in a solvent fraction eluting from the column. Most of the methanol was later evaporated from the collected solution giving an aqueous solution of each of the tritiated glyceryl nitrates.

Unlabelled Glyceryl Mono- and Di-Nitrates

The metabolite 1,2-GDN was synthesized from allyl alcohol using the procedure of Dunstan et al. (1965). 1,3-GDN was prepared by denitration of GTN by reaction with sodium nitrite (Dunstan et al., 1965). 1-GMN and 2-GMN was prepared from the partial nitration of glycerol (Dunstan et al., 1965). The purity of all metabolites was established by thin-layer chromatography (Crew and Di Carlo, 1968). Aqueous solutions of the glyceryl nitrates were assayed using the USP XX (1979) method.

4.2.2. High Performance Liquid Chromatography

Glyceryl Nitrates

A HPLC method based on that of Crouthamel and Dorsch (1979) was used for the analysis of tritiated GTN, 1,3-GDN, 1,2-GDN, 1-GMN and 2-GMN in supernatant buffer of resuspended erythrocytes or plasma. Aliquots of sample solutions were injected into a Waters Associates HPLC system equipped with a μ Bondapak C_{18} column. A mobile phase of water and methanol (70:30) was pumped at a flow rate of 1.0 ml/min. A Waters Associates Model 441 fixed wavelength detector set at 214 nm was used to identify the different glyceryl nitrates when their concentration was sufficient to allow their detection by the UV wavelength detector.

Samples (20-200μl) of buffer supernatant of erythrocyte suspensions, deproteinated plasma or plasma filtrate were injected directly into the HPLC system. Samples of plasma or tissue homogenates had been deproteinated with an equal volume of methanol. After centrifuging the sample tubes the clear supernatant was injected into

the HPLC system. With each injection of sample containing tritiated glyceryl nitrates, 5 µl of an aqueous solution (at least 10 µg/ml) of each of the unlabelled glyceryl nitrates was included in order to visualize the retention times of each of the compounds on the HPLC column using the UV detector. Collection of individual radio-labelled GDNs and mixed GMNs was facilitated by calculating the delay time between a peak exhibiting a UV response, and its efflux from the tubing draining the UV detector. For this calculation, the mobile phase flow rate, and the length and internal diameter of the HPLC tubing were used to calculate a delay time of 7 seconds.

Salicylates

The method of Rumble et al. (1981) was used to determine plasma, plasma filtrate or tissue homogenate concentrations of ASA, SA and SU. In essence this procedure involves injecting deproteinated plasma or tissue homogenates onto a C_{18} HPLC column. A mobile phase of 30% phosphoric acid, pH 2.5, in acetonitrile and a wavelength setting of 237 nm allows for the detection and quantitation of the above salicylates.

4.2.3. Liquid Scintillation Spectrometry

Tritiated glyceryl nitrates were quantitated by liquid scintillation spectrometry after HPLC separation. Fractions of the HPLC column eluant containing the eluting nitrates were collected into 15 ml plastic scintillation vials (Packard). Ten ml of scintillation cocktail (Biofluor, New England Nuclear) were added and samples were counted for their tritium content in a LKB 1215 Rackbeta 11 scintillation counter for 10 min or until 10,000 counts accumulated, which

ever occurred first. The counting efficiency was corrected in each sample by the external standard ratio method. Quench curves for ^{3}H in 10 ml scintillation cocktail had been determined using ^{3}H-toluene with carbon tetrachloride as the quenching agent. The efficiency values were plotted against the degree of quenching which was expressed in terms of the ratio of counts in 2 channels. The disintegration/min (dpm) for an unknown sample were then calculated, knowing the counting efficiency E and the counts/min (cpm), from the following expression:

$$dpm = \frac{cpm}{E} \qquad (4.1)$$

The amount of glyceryl nitrate in a given volume of plasma, plasma filtrate or buffer was determined from the specific activity of the ^{3}H-toluene. It was assumed that the glyceryl nitrate metabolites had specific activities per mole which were identical to that of the ^{3}H-GTN.

4.2.4. Gas Chromatography

Extractions and Evaporation Procedures

^{3}H-GTN was used extensively in the development of an assay of plasma GTN concentrations. Fresh blank sheep plasma was spiked with ^{3}H-GTN and extracted by 2 means:

(i) a rapid injection technique (Yap et al., 1978)

and

(ii) a vortexing technique.

In the injection technique, plasma (200 µl) was placed in a pointed glass test tube and rapidly injected with 200 µl hexane using a 1 ml glass syringe and 21 gauge needle. After injection the hexane was removed from the plasma to a scintillation vial for counting of tritium content. This process was repeated 11 times and the hexane extracts added to 11 separate scintillation vials for counting.

The vortexing technique involved adding hexane to spiked sheep plasma samples (200 µl) in a final volume ratio of either 3:1 or 6:1. The glass tubes were sealed with glass tops and the tubes were vigorously vortexed for 1 min while ensuring that hexane did not touch the top and so escape through the glass/glass interface. Hexane was removed into scintillation vials for counting. The process was repeated 4 times.

In both extraction techniques the tritium content of each volume of hexane used to extract GTN from plasma was compared to the tritium content of a 200 µl aliquot of the pre-extracted plasma and the recoveries of the drug from plasma were determined.

The effect of evaporating the hexane in the pooled extracts on the recovery of GTN was also determined using ^{3}H-GTN. Spiked plasma samples were extracted with hexane and the pooled extracts were slowly evaporated under a gentle stream of high purity nitrogen. The nitrogen was delivered from a cylinder (C.I.G. Australia) connected via metal gas lines through a hydrocarbon trap (Model GFMSX, S.E.G., Melbourne). The hexane was evaporated to about 20 µl and an aliquot was assayed for tritium content and compared to the tritium content of an aliquot of the pre-evaporated hexane extracts to determine the loss of GTN during the evaporation step.

In the extraction technique used in this work 200 µl plasma was mixed, by vortexing for 1 min, with 1200 µl hexane twice and the hexane supernatant was aspirated and pooled. An internal standard, isosorbide dinitrate (ISDN) in hexane (100 µl), was then added to the extract. Three ISDN stock solutions of different strengths (0.6, 6, 15 µg/ml) were used to meet the varying GTN plasma concentrations being analyzed. A preliminary analysis was used to ascertain the appropriate internal standard concentration required. The extent of evaporation of the hexane eluants was adjusted for the approximate GTN concentration present to ensure that less than 1 ng GTN was injected on column and the electron capture detector did not become saturated. The final volumes of hexane after evaporation ranged from 20 to 250 µl.

Separation and Quantitation

Aliquots (2-5 µl) of the evaporated hexane extracts were injected onto a 2 M glass column (i.d. 2 mm) packed with 3% SP 2401 on 100/120 mesh (Supelco) in a Shimadzu Model 6A GC equipped with a ^{63}Ni electron capture detector. The column was prepared by silanizing and then being connected in series with a pre-column containing 3% Carbowax 20 M-TPA on Diatomite 70-80 mesh. The temperature of the oven was raised to 300°C at which temperature degradation of the packing occurs and the decomposition products were deposited on the walls of the empty silanized column, which was downstream of the pre-column (with relation to gas flow), coating any remaining active sites (Taylor et al., 1981). The prepared column was then packed with 3% SP 2401 and conditioned at 200°C overnight.

The operating conditions of the GC assay were; column temperature 145°C, injection and detector temperature 180°C, nitrogen carrier gas

flow rate 80 ml/min At the start of each day's operation, the column was primed by 2 injections of 0.5 ng GTN in 2 µl hexane.

4.3 IN VITRO METABOLISM AND DISTRIBUTION

4.3.1 Sources and Preparation of Tissues

Blood

Human and sheep venous blood was collected from three human volunteers and three sheep into heparinized plastic tubes which were centrifuged at 1100 rpm for 15 min. The plasma was removed and stored at 4°C until required. The erythrocytes were washed in a volume of buffer (equal to the volume of plasma removed) consisting of 10 mM KCl, 10 mM glucose and 20 mM Tris-HCl, pH 7.4 (Mircevova et al., 1977). Packed erythrocytes were obtained following centrifugation at 1100 rpm for 15 min and were then resuspended in a fresh volume of buffer. The haematocrit was maintained at 0.45 by adjustment of the buffer volume.

Other Tissues

Pieces of liver, lung, leg muscle, vena cava and aorta were removed from three sheep anaesthetized with sodium pentobarbitone. The pieces of tissue were placed in ice cold 0.1M phosphate buffer at pH 7.4, weighed and briefly dried on blotting paper. After slicing into small pieces, the tissues were homogenized in two volumes of fresh phosphate buffer in a Sorvall blender to form a final tissue suspension. The protein content of each suspension was measured by the method of Lowry et al. (1951). The mean protein content (mg/ml) of

tissue homogenates was liver, 28.5; lung, 8.5; muscle, 15.8; vein, 2.7; artery, 1.5.

4.3.2. Tissue Incubations With Nitroglycerin

Human Studies

Blood. In initial studies, a known volume (10-50 µl) of buffer containing known amounts of GTN, 1,3-GDN and 1,2-GDN, was added to erythrocyte suspensions (6-8 ml) in 10 ml silanized glass centrifuge tubes pre-heated to 37°C in a water bath. The resulting GTN concentrations ranged from 100 µg/ml to 50 ng/ml suspension while the GDN concentrations ranged from 60 to 1 µg/ml suspension. After mixing by inversion for 1 min aliquots (200 µl) of resuspended erythrocytes were removed into chilled, silanized glass centrifuge tubes containing 20 µl iodoacetamide (final concentration of 4 mM). Thereafter aliquots were removed at known time intervals. Samples were immediately centrifuged and the supernatant buffer was analyzed for GTN and its metabolites using HPLC (see Section 4.2.2.).

The results of those initial experiments (see Section 5.2.1.) showed the "zero-time" buffer GTN concentrations to be always less than intended initial GTN concentrations calculated using the known amount of GTN added to the known volume of erythrocyte suspension. In order to accurately quantitate this initial rapid uptake of GTN (apparently into the erythrocytes) the method of preparing GTN incubations was changed.

Blood and resuspended erythrocytes (8 ml) were pipetted into 10 ml glass centrifuge tubes which were placed in a water bath at 37°C for

15 min. The blood and suspension were centrifuged and the supernatant plasma or buffer quickly removed. Equivalent volumes of plasma or buffer pre-heated to 37°C and containing specified amounts of tritiated glyceryl nitrates were then added. The amounts of the tritiated GTN, 1,2-GDN or 1,3-GDN added resulted in final GTN concentrations in the range of 600 to 0.8 ng/ml and separate 1,2-GDN and 1,3-GDN concentrations of about 20 ng/ml. The reconstituted blood and erythrocyte suspension containing GTN or GDNs were thoroughly mixed for 5 sec on a vortex mixer and aliquots (0.5 ml) were removed at frequent intervals and placed into glass tubes containing 20 µl iodoacetamide (final concentration of 4 mM). These samples were centrifuged at 3000 rpm for 3 min and the supernatant plasma or buffer removed for analysis. Drug concentrations in the spiked stock plasma or buffer solutions were used to quantitate the rapid fall in the GTN, 1,2-GDN and 1,3-GDN concentrations on the addition of erythrocytes. The decline in drug concentrations in plasma and buffer allowed calculations of apparent erythrocyte/plasma and erythrocyte/buffer partition coefficients.

Plasma. In initial studies investigating the metabolism of GTN by human plasma, unlabelled GTN in 10-50 µl buffer was added to plasma to give final GTN concentrations in the range 50 µg/ml to 50 ng/ml plasma. After mixing, aliquots were removed for zero-time GTN analysis (and thereafter at known time intervals) by HPLC (see section 4.2.2.). In later experiments labelled GTN was used to study the metabolism of GTN in plasma at concentrations of 50 and 10 ng/ml plasma.

Inhibition of GTN Metabolism by GDN. The inhibition of GTN metabolism by 1,2-GDN and 1,3-GDN in erythrocytes was tested by adding unlabelled

1,2-GDN and 1,3-GDN in varying concentrations to the buffer containing the GTN. Final GDN concentrations ranged from 0 to 600 ng/ml and a GTN concentration of about 10 ng/ml was used.

Inhibition of GTN Metabolism by Iodoacetamide. The inhibition of GTN metabolism in blood by iodoacetamide was studied by adding labelled GTN to pre-warmed plasma to give a final GTN concentration in reconstituted blood of about 1.5 ng/ml. After rapid mixing of plasma and packed erythrocytes, blood samples were removed for analysis of plasma GTN concentrations in order to monitor the loss of GTN at 37°C. After 6 min of incubation at 37°C the contents of the centrifuge tubes were divided into two equal volumes and each volume was placed in a glass centrifuge tube pre-cooled to 2°C. One tube contained 100 µl of an aqueous iodoacetamide solution (final concentration 4mM) and the other tube contained 100 µl buffer. Aliquots (500 µl) were removed from each of the cooled tubes after 3 min and thereafter at known time intervals to determine the plasma GTN concentration.

Sheep Studies

Blood. The in vitro metabolism of GTN by sheep blood, resuspended erythrocytes and plasma was studied using the procedures described above for human blood components. Initial GTN concentrations in resuspended erythrocytes and plasma were 50 and 10 ng/ml. A GTN blood concentration of 1.5 ng/ml was used to investigate inhibition of GTN metabolism by iodoacetamide. The metabolism of 1,2-GDN and 1,3-GDN (23.6 ng/ml and 20.7 ng/ml respectively) by sheep blood and resuspended erythrocytes was also investigated.

Other tissues. Aliquots (4 ml) of the sheep liver, lung, leg muscle, vena cava and aorta homogenates were transferred to glass centrifuge tubes in a water bath heated to 37°C. As recommended by Needleman and Hunter (1965) the following co-factors were added to the homogenates; reduced glutathione, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADPH. Tritiated GTN was added in 100 µl saline to give concentrations of about 50 and 10 ng/ml suspension. GTN was also added to tubes containing buffer and co-factors but no tissue. The possibility of metabolite inhibition of GTN metabolism was studied by adding 1,3-GDN and 1,2-GDN (each at final concentrations of about 100 ng/ml suspension) to some of the tubes.

At specified time intervals 200 µl aliquots were removed from the homogenates incubated at 37°C. The inhibition reaction was terminated in 200 µl samples by the addition of 200 µl methanol. After vortexing and centrifugation the samples were stored at -20°C until analyzed.

4.3.3. Incubations With Aspirin

ASA was incubated with homogenates of liver, lung and leg muscle at 37°C in a manner similar to that described in Section 4.3.2. However, no co-factors were added to the homogenates in these studies. Incubations were also performed using buffer only. In tissue and buffer incubations, initial ASA concentrations of 50 and 5 µg/ml were employed. SA, at a final concentration of about 50 µg/ml homogenate, was added to some of the homogenates. The incubation reaction was terminated by addition of 20 µl of 3% perchloric acid and 200 µl methanol. After vortexing and centrifugation the samples were stored at -20°C until assayed.

4.3.4. Plasma Protein Binding

The unbound fraction of GTN, 1,2-GDN, 1,3-GDN, ASA, SA and SU was determined using an ultrafiltration technique (Amicon Micropartition System with YMT membranes). Control experiments using aqueous solutions of the unlabelled drugs (except SU) were performed to assess whether any sorption to the plastic apparatus and membranes occurred.

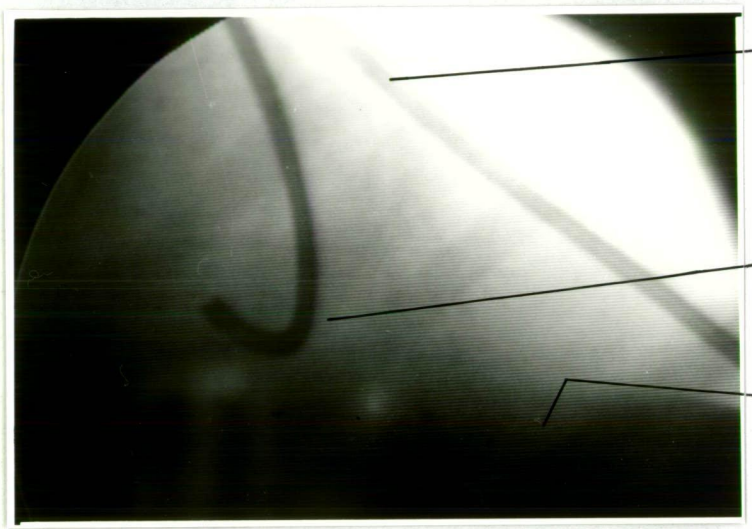
Glyceryl Nitrates. Blank human and sheep plasma (1 ml) pre-warmed to 37°C and then spiked with labelled drug was loaded into the donor side of the ultrafiltration apparatus (previously equilibrated at 37°C in an oven). The apparatus was then centrifuged at 3000 rpm for 10 min at 37°C. Aliquots of the pre-centrifuged plasma (total) and filtrate (free) were assayed for glyceryl nitrate content by HPLC and liquid scintillation spectrometry as described in Section 4.2.

Salicylates. Blank sheep plasma was used for in vitro experiments to investigate the binding of ASA and SA to plasma proteins under various simulated experimental conditions. Conditions studied were plasma dilution, the presence of pentobarbitone (anaesthetic) and potassium fluoride (for inhibition of ASA hydrolysis in vitro). Salicylates (ASA and SA) in 0.1M phosphate buffer pH 7.4 (10 µl) were added to glass tubes containing 10 µl potassium fluoride in buffer (final concentration 2.5 mg/ml) and 10 µl pentobarbitone in saline (final concentration 10 µg/ml). Pre-warmed (37°C) plasma or dilute plasma (1:1 in buffer) were added to give final ASA concentrations of 10 or 50 µg/ml and SA concentrations of 0, 10 and 50 µg/ml in various combinations. Samples of the plasma (1 ml) were thoroughly mixed by vortexing for 1

(a)

Balloon of Swan-Ganz catheter in pulmonary artery

(b)

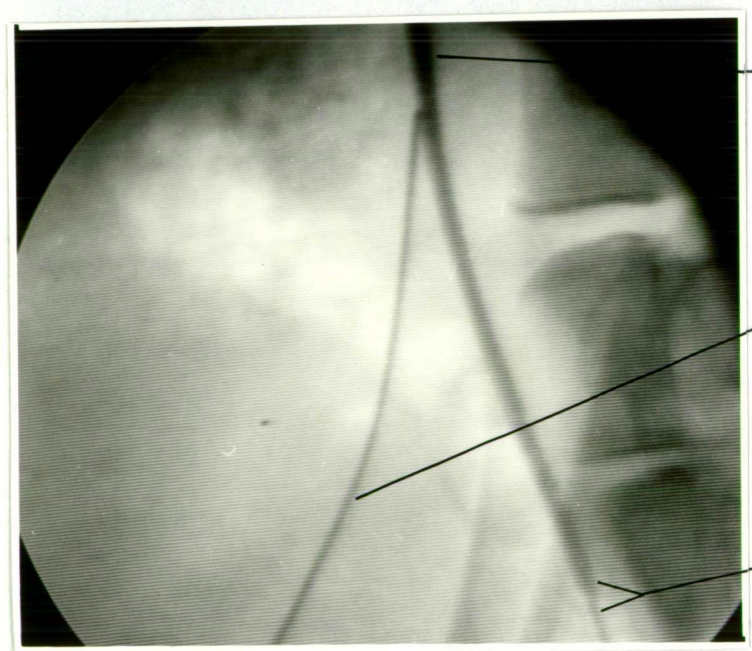


Catheter in vena cava

Catheter in hepatic vein

Liver

(c)



Catheter in vena cava

Guiding wire in non-radio opaque infusion tubing in right femoral vein

Guiding wire and catheter in left femoral vein

Figure 4.1 Fluoroscopic images of blood sampling catheters in the (a) pulmonary artery, (b) Hepatic vein and (c) femoral vein.

min and loaded into the donor side of the ultrafiltration devices, previously equilibrated at 37°C in an oven. The devices were then centrifuged at 3000 rpm for 15 min at 37°C. Aliquots of the pre-centrifuged plasma (total) and filtrate (free) were assayed for salicylate content by HPLC as in 4.2.2.

4.4. PHARMACOKINETICS AND PHARMACODYNAMICS OF NITROGLYCERIN

4.4.1 Preparation of Sheep.

Sheep, with weights ranging from 25 to 49 kg, were anaesthetized with sodium pentobarbitone (Nembutal, Abbott Laboratories) using a dose of 90 mg/kg injected into a jugular vein, and maintained under anaesthesia by a continuous intravenous infusion of sodium pentobarbitone at a rate of 10 mg/hr/kg. The pulmonary artery was visualized via a left thoracotomy at the level of the 3rd rib. A Narco Biosystems electromagnetic flow transducer was placed around the pulmonary artery and blood flow (cardiac output) was measured on a Narcomatic electromagnetic flow meter.

Drug solutions were administered through polyolefin tubing (Tridil, American Critical Care) into the right femoral vein of the sheep. Blood samples were withdrawn from sites immediately before and after the liver (portal and hepatic veins), before and after the lungs (pulmonary artery and left ventricle) and before and after the left hind leg (femoral artery at the bifurcation of the aorta, and femoral vein). The stomach was exposed via a left lateral incision and a catheter was placed in the portal vein via a large gastric vein. Using fluoroscopy, a catheter was inserted into the hepatic vein via a jugular vein (see Figure 4.1.(b)).

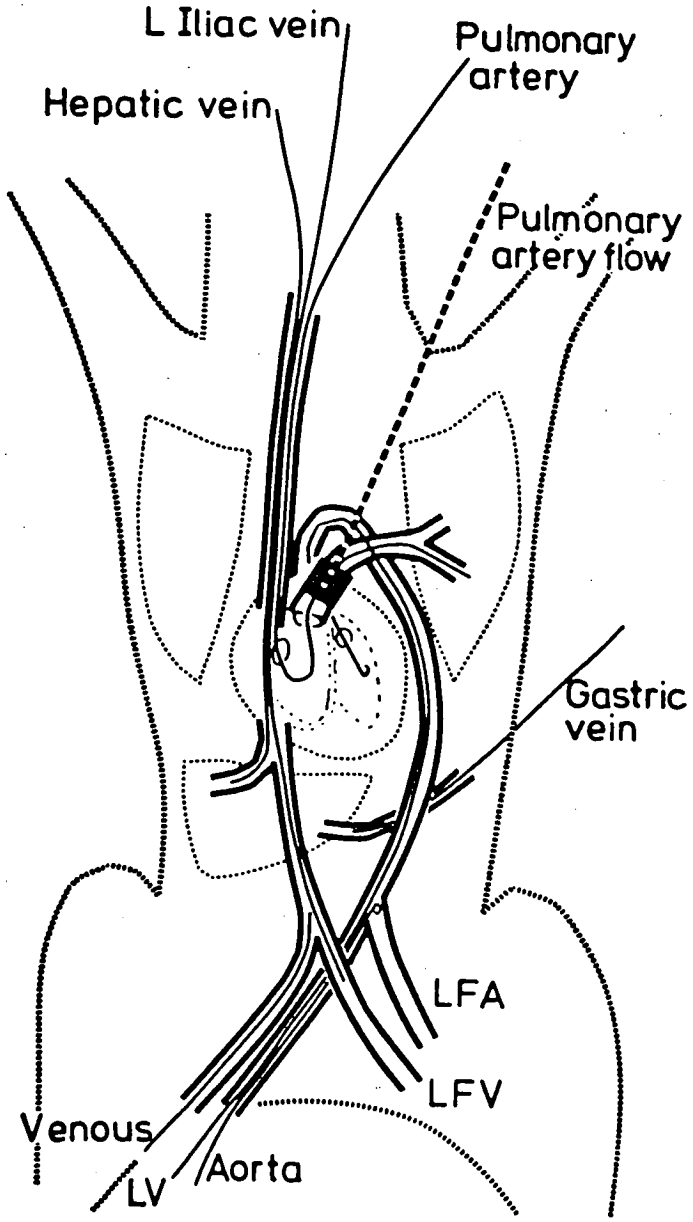


Figure 4.2 Schematic representation of the blood sampling sites in sheep receiving intravenous infusions.
LV=left ventricle; LFV=left femoral vein; LFA=left femoral artery.

Pre-lung blood samples were taken from the pulmonary artery via a Swan-Ganz catheter inserted into a jugular vein and guided into the pulmonary artery using fluoroscopy (See Figure 4.1.(a)). Post-lung samples were taken from a catheter in the left ventricle which was inserted via a femoral artery and which was also used to measure left ventricular pressure.

Pre-hind leg blood samples were taken from a catheter placed in the aorta at the bifurcation and which was inserted via a femoral artery. The catheter was also used to measure arterial blood pressure. Post-hind leg blood samples were taken from a catheter in the left femoral vein which had been inserted via a jugular vein using fluoroscopy (see Figure 4.1.(c)).

A schematic representation of the blood sampling sites in the sheep is presented in Figure 4.2. Baseline blood flow in the femoral artery was measured in two of the later experiments by placement of an electromagnetic flow transducer around a femoral artery.

Each sheep received a continuous intravenous infusion of normal saline at a rate of about 1 ml/min into a jugular vein throughout the course of an experiment. At the completion of most of the sheep experiments, the liver and associated major blood vessels were exposed and examined in situ to corroborate the fluoroscopic evidence that blood sampling lines were indeed in the correct vessels.

4.4.2. Bolus Intravenous Infusion Dosing Schedules

Solutions of GTN were made by dissolving the appropriate number of Anginine tablets (Burroughs Australia) in normal saline and filtering before use. For the largest concentrations of GTN used and where the solubility of GTN in saline was exceeded, ethanolic solutions of GTN

(Tridil, American Critical Care) were made up in saline. In an initial study, doses of GTN (2.7, 5.4, 13.6, 27.2, 54.4, 108.8 μg/kg) were administered in 15 ml saline and were injected, over 1 min, into the left femoral vein of three sheep. At least 20 min elapsed between administering each bolus dose. Five seconds prior to completing the bolus dose, blood samples were withdrawn from the femoral artery and femoral vein for analysis of 'zero-time' plasma GTN concentrations. Thereafter blood samples were withdrawn at 1, 2, 3, 4, 5, 6, 8, 10, 12 and 15 min for analysis of plasma GTN concentrations. Sampling across the liver and lungs was not possible during these experiments as insufficient time existed to collect all samples rapidly during the fast elimination phase of GTN in vivo.

Arterial blood pressures and left ventricular pressures (measured against 100 cm H_2O) were monitored continuously during these experiments as was cardiac output.

4.4.3. Continuous Intravenous Infusion Dosing Schedules

GTN Pharmacokinetics

Doses of GTN in saline were infused from a 60 ml polyethylene syringe (Terumo Australia) via polyethylene tubing inserted into the left femoral vein. Previous studies had shown that the availability of GTN infused through polyethylene tubing was about 96% (Cossum et al., 1978). The syringe was compressed by a syringe pump (Sage Instruments, Model 355) to give a flow rate of infusion solution of 0.99 ml/min. This flow rate, with appropriate GTN concentrations in saline, was used to produce rates of GTN infusion of 0.4, 5.7 and 22.1 μg/min/kg sheep with any one dose being delivered to three sheep. Infusions lasted for

40 min during which time blood samples were withdrawn from the portal and hepatic veins, pulmonary artery and left ventricle, and femoral artery and femoral vein. On cessation of the infusion blood samples were taken from the femoral artery and femoral vein for up to 25 min for analysis of plasma GTN concentrations. (Preliminary studies suggested that the disappearance of GTN was too rapid to allow for blood sampling from all available sites).

Two of the 3 GTN infusions at the rate of 0.4 μg/min/kg were performed using ^{3}H-GTN in order to follow the time course of 1,3-GDN, 1,2-GDN and mixed GMNs. After terminating the infusion at 40 min, blood samples were taken during the next 90 min from all sampling sites (portal and hepatic veins, pulmonary artery and left ventricle, femoral artery and femoral vein) for the analysis of plasma concentrations of the tritiated glyceryl nitrates.

Effects of GDNs on GTN Pharmacokinetics

Continuous GTN infusions (5.7 μg/min/kg sheep) were commenced in two sheep and blood samples taken from all sampling sites as described above. After 30 min of GTN infusion, a bolus dose of 1,3-GDN and 1,2-GDN (total 5 mg in 15 ml saline) was infused into the aorta over a period of 1 min. The GTN infusion was maintained for a further 5 min in one sheep and 10 min in the other sheep after which blood samples were withdrawn from the femoral artery and femoral vein at 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 20 and 25 min post-infusion for analysis of plasma GTN concentrations.

Arterial blood pressure and cardiac output were monitored continuously during these experiments.

4.4.4. Collection of Blood Samples

All blood samples (2 ml) were collected in silanized glass syringes and quickly transferred to chilled silanized glass tubes containing heparin and 10 µl of a solution of iodoacetamide in saline (final concentration 4 mM). The contents of the tubes were mixed by inversion before centrifuging at 3000 rpm for 3 min. The resulting plasma was transferred to silanized glass tubes, using silanized glass pipettes, and stored at -20°C until the samples were analyzed for their glyceryl nitrate content, which always occurred within two days of an experiment being performed.

4.4.5. Stability of Glyceryl Nitrates in Plastic Catheters

Simulated experiments were performed to assess the extent of sorption of organic nitrates by the plastic catheters used in the work for blood sampling. Labelled glyceryl nitrates in normal saline were infused from glass beakers through plastic catheters using a glass syringe. Flow rates of 5 and 40 ml/min were used for GTN solutions and 40 ml/min was used for the other drugs.

4.5. PHARMACOKINETICS AND PHARMACODYNAMICS OF ASPIRIN

4.5.1 Studies in Sheep

Preparation of Sheep

Sheep were prepared in the same manner as described in Section 4.4.1. for GTN experiments.

Continuous Intravenous Infusion Dosing Schedules

ASA (Aspro Clear, Nicholas Pty. Ltd.) was dissolved in saline to give appropriate ASA concentrations for infusion (pH 5.9). Solutions were infused in the same manner described for GTN in Section 4.4.3. but at a flow rate of 0.81 ml/min. This flow rate, with appropriate ASA concentrations in saline, was used to produce rates of ASA infusion of 61 and 485 µg/min/kg sheep.

Four sheep received an ASA infusion at the rate of 61 µg/min/kg. Blood samples were taken from portal and hepatic veins, pulmonary artery and left ventricle, and femoral artery and femoral vein. Infusions of ASA lasted for 75 min. Three of the four sheep also received, at the 50th min of ASA infusion, a bolus dose of NaSA in 15 ml saline administered over 1 min into the aorta. One sheet received a bolus dose of NaSA equivalent to 30 mg SA, another sheep a dose equivalent to 300 mg SA and the third sheep a dose equivalent to 1200 mg SA. At the termination of the ASA infusion after 75 min, blood samples were taken from the femoral artery and femoral vein at 5, 10, 20, 30, 45, 60, 75 and 90 min post-infusion. Some blood samples were also taken from liver and blood vessel sampling sites during that time.

Four other sheep received an ASA infusion at the rate of 485 µg/min/kg. Blood sampling was performed as described for the 61 µg/min/kg infusions, however, no bolus doses of NaSA were administered to these sheep.

Collection of Blood Samples

Blood samples (2 ml) containing salicylates were collected into chilled glass tubes containing heparin and 50 µl potassium fluoride solution in normal saline (final concentration in blood, 5 mg/ml)

(Rowland and Riegelman, 1967). Samples were centrifuged at 3000 rpm for 10 min and the resulting plasma was removed and stored at -20°C until analyzed for salicylate content using the HPLC assay of Rumble et al. (1981).

4.5.2. Studies in Man

Single Dose Study

After overnight fasting, nine healthy male volunteers, aged between 19 and 24 years and weighing between 67 and 79 kg, received, with 200 ml water, equimolar oral doses of salicylate (230 mg) in the form of soluble ASA, NaSA and an enteric coated and microencapsulated formulation of ASA at different times. An interval of at least two weeks elapsed between the administration of each dose-form to the same subjects. Blood samples were collected immediately before ingestion of the dose and at fixed times thereafter.

Continuous Dose Study

Sixteen healthy volunteers (eight male and eight female) were used. The ages of volunteers ranged from 20 to 30 years (average 22.6 years) and with body weights ranging from 54 to 105 kg, (average 68.8 kg). The study was conducted in a cross-over manner using two groups of eight subjects, each subject ingesting three different doses of either 20, 50, 100, 200, 650 and 1300 mg ASA in the slow release formulation. The appropriate amount of microencapsulated drug granules were weighed out and placed in gelatin capsules. Each group of eight subjects consisted of four males and four females. A group of males (4) only, taking either 20, 50, 100 or 200 mg doses and all subjects in

the groups taking 650 and 1300 mg were used specifically to determine the degree of inhibition of platelet function induced by AA.

Control values of platelet aggregation and MDA production were estimated before the commencement of each dosing schedule. Subjects ingested one capsule of a given dose of the slow release product each morning for six days. On the seventh morning, blood samples were taken for assessment of trough values of platelet function and plasma ASA concentrations. Subjects ingested the last capsule and 3 hr later peak values of platelet function and plasma ASA concentrations were determined. This time was chosen to correspond with the time (3 hr) to reach mean peak plasma ASA concentration determined in the single dose study (Fig. 5.63.).

Subjects in both groups were not permitted to take any drugs for two weeks prior to the study or during the study. The experiments were performed according to the principles of the Declaration of Helsinki. Approval was obtained from the University of Tasmania Committee on ethical aspects of human biological research, and informed written consent was obtained from each subject.

Collection of Blood Samples

Blood was collected into a plastic syringe either by venepuncture (18-19 G needles) or from an indwelling catheter (Jeltec 17-18 G) in a forearm vein. The blood was immediately distributed to tubes containing anticoagulants and /or potassium fluoride for the appropriate determination. Blood for platelet function tests was used within 3 hr.

Assays of Platelet Function

Platelet Aggregation. Platelets from 9 ml blood mixed with 1 ml 3.8% sodium acid citrate solution were obtained by centrifugation at 190 g for 15 min to produce platelet rich plasma (PRP). Further centrifugation at 2,000 g for 15 min gave platelet poor plasma (PPP). Platelet counts for PRP were obtained as before and PRP was diluted with PPP to give final platelet counts of 200-250 x 10^9 per litre. Platelet aggregation was measured by the method of Born (1962) using a Chronolog aggregometer. Aggregation of 450 µl PRP was initiated by adrenalin (2.5 µM) and collagen (2.5 µg). AA (0.87 mM) was also used in some experiments. Inhibition of platelet aggregation was measured as the decrease in light transmission and expressed as the percentage of control aggregation.

MDA Production. MDA production induced by stimulation of PRP with NEM was measured spectrophotometrically by a modified method of Catalano et al., (1981). PRP was obtained from 7.5 ml blood collected into 1 ml 3.8% w/v sodium edetate. Saline (1.5 ml) was added and the mixture centrifuged at 190 g for 15 min. Two ml of the resultant PRP were added to 5 ml saline and centrifuged at 2000 g for 10 min. The supernatant was decanted and the tube inverted and drained onto absorbent paper for 2 min. The platelet button so obtained was resuspended in 900 µl 14 mM Tris buffer (pH 7.4) and incubated for 15 min with 0.1 100 µl 50 mM NEM or 100 µl 2 mM AA. The reaction was stopped by the addition of 1 ml of 20% trichloracetic acid in 0.6 M HCl and cooled in ice for 5 min. The mixture was centrifuged at 2000 g for 15 min and 1.5 ml of the clear supernatant was mixed with 300 µl of 0.13 µl TBA

reagent and heated in a water bath at 70°C for 3 min using marbles as condensers. The TBA reagent was prepared by dissolving 1.73 g TBA in 0.25 M Tris buffer, adjusting the pH to 7 with 10 M HCl and filtering through a number 3 sintered glass funnel. The reaction mixture was cooled to room temperature and the absorbance of the pink chromagen was measured at 532 nm on a Pye Unicam SP6-550 spectrophotometer against a control from PRP with NEM or AA. Platelet counts were made on a Thrombocounter C (Coulter Electronics Ltd.) and MDA production was calculated as nanomoles per 10^9 platelets.

4.6. TREATMENT OF DATA

4.6.1. Pharmacokinetic Parameters

The elimination rate constant k was estimated by linear regression of the second phase of the post-infusion log drug plasma concentration versus time plots.

The area under the plasma drug concentration versus time curve (AUC) was estimated from the sum of the $AUC_{0->t}$ (where t is the time of the last data point) and $AUC_{t->\infty}$. $AUC_{0->t}$ was obtained using the trapezoidal rule on a desk top computer. $AUC_{t->\infty}$ was calculated by the equation:

$$AUC_{t->\infty} = C_t/k \qquad (4.2)$$

where C_t = concentration of drug at time t and k = elimination rate constant.

The area under the first moment curve (AUMC) is defined by the area under a plot of the product of concentration and time versus time.

This area from time zero to the time t of the final data point was calculated using the trapezoidal rule on a desk top computer. $AUMC_{t->\infty}$ was calculated from the following equation (Gibaldi and Perrier, 1982):

$$AUMC_{t->\infty} = \frac{tC_t}{k} + \frac{C_t}{k^2} \qquad (4.3)$$

Mean residence time (MRT), the first moment, was calculated as the ratio AUMC/AUC (Gibaldi and Perrier, 1982) and the MRT of the solute in an organ was calculated as the difference of the arterial MRT and venous MRT.

Availability of a drug is calculated as the ratio of the amount of drug leaving an organ or system to that entering. Thus, organ availability (F_o) was calculated from the venous and arterial AUCs:

$$F_o = \frac{AUC_{venous}}{AUC_{arterial}} \qquad (4.4)$$

and the systemic availability (F) from dose (D) and AUC (venous unless specified otherwise):

$$F_o = \frac{D}{AUC} \qquad (4.5)$$

The fraction of substance unbound (f_u) was calculated from the following equation:

$$f_u = \frac{C_u}{C} \qquad (4.6)$$

where C_u is the unbound plasma drug concentration and C the total plasma drug concentration.

Intrinsic clearance (Cl_{int}) of all substances was calculated from:

$$Cl_{int} = \frac{Q\ (1 - F)}{f_u \times F} \qquad (4.7)$$

where Q = blood flow to the organ, F = availability, and f_u = unbound fraction of the drug in plasma. Equation 4.7 is a rearrangement of the equation used to relate clearance, blood binding and blood flow assuming a "well-stirred" model (Gibaldi and Perrier, 1982). As blood flows to individual organs were not routinely monitored, average estimated values were employed. Blood flows to the lungs and liver were assumed to be 1.0 and 0.25 time cardiac output respectively. A blood flow of 0.1 times cardiac output was used for calculation of hind-leg intrinsic clearance based on results of the baseline blood flow measurements in the femoral artery of two sheep. Average data were also used for binding in calculations of intrinsic clearance. The fraction unbound, f_u, for GTN, 1,2-GDN and 1,3-GDN were assumed to be 0.39, 0.77 and 0.89 respectively. The fraction unbound for salicylates was based on individual data obtained. The protein binding of salicylates was not monitored in two sheep given 485 µg/min/kg of ASA. The mean f_u data from the other sheep given this dose were therefore used to calculate intrinsic clearances for these sheep.

Apparent erythrocyte/plasma and erythrocyte/buffer partition coefficients were calculated as the ratio of the GTN concentrations in erythrocytes and the GTN concentration in plasma or buffer. The partition coefficients (k) were determined using equation 4.8.

$$K = \frac{A_1 - C_o V_T}{C_o V_E} \qquad (4.8)$$

Where A_i is the amount of GTN added to volume V_T of erythrocyte suspension, V_E is the volume occupied by the erythrocytes and C_o is the concentration of GTN in buffer or plasma immediately on mixing. C_o was estimated by back extrapolating the terminal phase of the plot of log concentration versus time to zero-time.

4.6.2. Statistical Analysis

Data are presented as mean ± S.E. Statistical comparisons between groups were performed using Students t-test or analysis of variance. A p value of ≤ 0.05 was taken as statistically significant.

CHAPTER 5

RESULTS

5.1 ANALYTICAL TECHNIQUES

5.1.1. Analysis of Glyceryl Nitrates

Synthesis of Glyceryl Nitrates

Figure 5.1 shows the time course of GTN yield using the reaction conditions outlined in 4.2.1. The greatest yield of unlabelled GTN under the conditions used occurs between 45 and 60 min of incubation. The incubation time of 30 min used in the synthesis of ^{3}H-GTN resulted in the yield of ^{3}H-GTN being reduced to about 50% but enabled adequate quantities of ^{3}H-GDNs to also be synthesized.

Purification of the reaction products gave ^{3}H-GTN with a specific activity of 0.125 Ci/mmol (2100 dpm/ng, in September, 1982) and radiochemical purity of 98.9%. ^{3}H-GTN was stored in aqueous solution (40 µg/ml) in glass vials at -20°C until used.

The specific activity of the ^{3}H-GDNs was assumed to be the same as that of ^{3}H-GTN on a molar basis (2619 dpm/ng, in September, 1982).

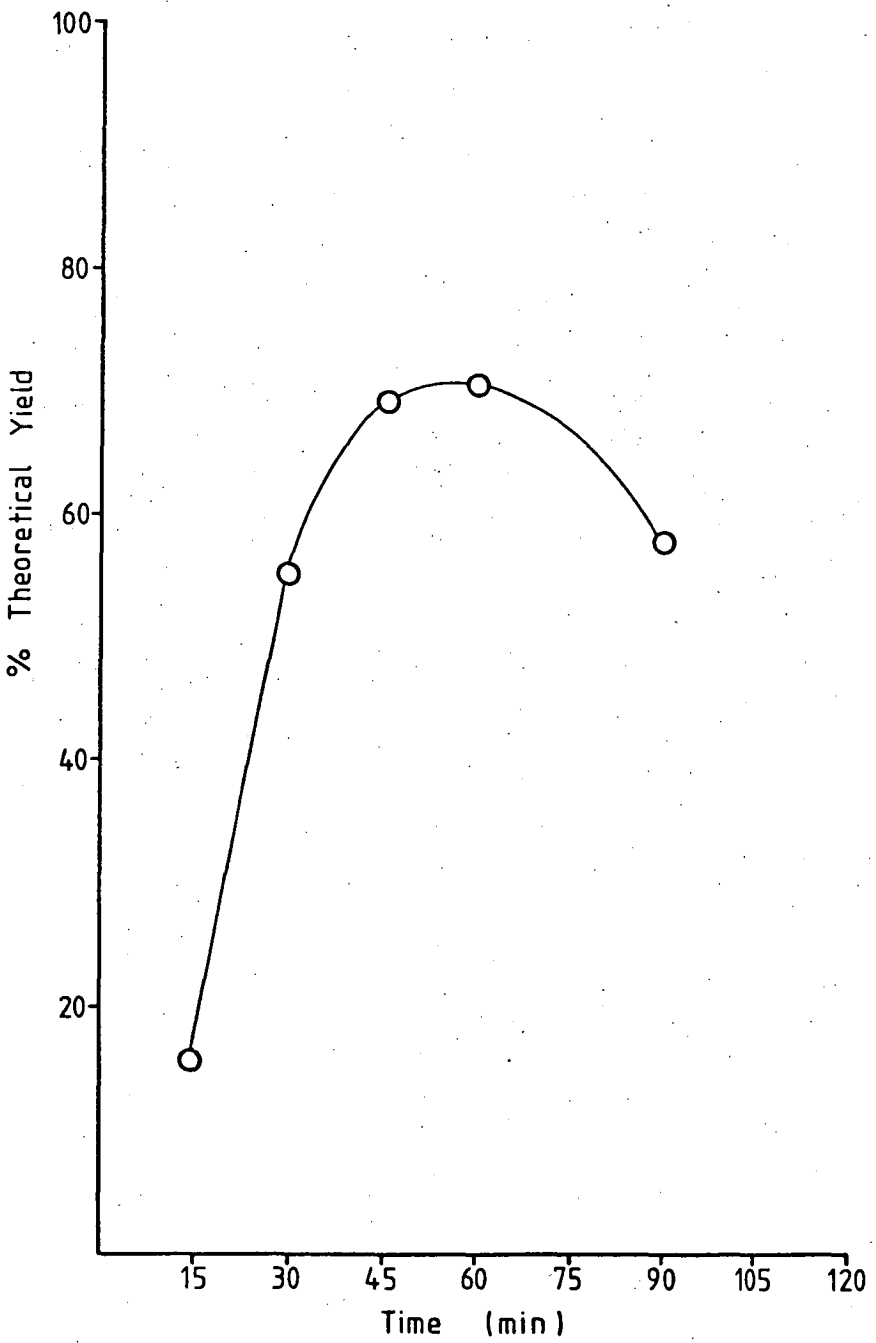


Figure 5.1 Optimization of the yield of nitroglycerin from incubations of glycerol, fuming nitric acid and fuming sulphuric acid under the conditions described in Section 4.2.1.

Table 5.1

Comparison of recoveries of labelled nitroglycerin (GTN), 1, 3- and 1, 2- dinitroglycerins (1, 3- GDN, 1, 2- GDN) and mixed mononitroglycerins (GMN) from a C_{18} HPLC column with and without a similar but unlabelled compound added as a marker of retention time. (mean ± S.E.)

Drug	Concentration (ng/ml plasma)	% Recovery with marker (n = 5)	% Recovery without marker (n = 5)
GTN	0.8	95.9 ± 0.6	73.0 ± 1.2
	10.0	99.2 ± 1.0	79.5 ± 1.8
	98.8	99.4 ± 0.7	78.4 ± 1.0
1, 3- GDN	0.5	99.5 ± 0.6	84.5 ± 1.6
	10.5	99.7 ± 0.3	86.2 ± 2.0
	115.0	98.9 ± 0.3	89.8 ± 1.0
1, 2- GDN	0.5	97.8 ± 0.5	86.0 ± 1.1
	10.0	99.1 ± 0.7	87.5 ± 0.8
	112.0	99.9 ± 1.2	89.1 ± 0.7
GMN's	0.6	98.8 ± 0.4	87.7 ± 1.1
	11.0	99.4 ± 0.6	87.4 ± 1.7
	92.7	100.0 ± 0.7	89.2 ± 0.5

Table 5.2 Recoveries of labelled glyceryl nitrates from plasma by deproteination with methanol. (Mean ± S.E. (n = 3)).

Drug	Drug concentration (ng/ml)	% Recovery
GTN	1.1	99.2 ± 0.07
	10.7	99.9 ± 0.06
1, 3-GDN	5.0	97.1 ± 0.21
1, 2-GDN	4.6	100.0 ± 0.03
GMNs	9.9	99.6 ± 0.08

High Performance Liquid Chromatography

Retention times for injections of 20 µl solution of glyceryl nitrates on the HPLC system used were as follows:

1-GMN 3 min, 2-GMN 3.6 min, 1,3-GDN 6 min

1,2-GDN 7 min, GTN 20 min (Figure 5.2 (a)).

Increasing the injection volume of 150 µl affected the chromatographic behaviour of the glyceryl nitrates (Figure 5.2(b)). At the larger injection volume the GMNs cannot be satisfactorily resolved.

In initial studies plots of dpm versus concentration (range 0.2-100 ng/ml, ^{3}H-GTN and ^{3}H-GDNs, 150 µl injections) were non-linear. Addition of unlabelled GTN and GDNs (5µl of aqueous glyceryl nitrate solution containing 10-50 µg/ml) was required in each 150 µl injection of deproteinated plasma or buffer containing labelled drug to obtain linear calibration plots for the labelled drugs. Recovery experiments for labelled drugs in the concentration range 0.5-115.5 ng/ml plasma (or buffer) showed that the poor linearity of calibration plots was due to a poor recovery of small amounts of labelled drug from the chromatography column (Table 5.1).

Deproteination of plasma with methanol resulted in recoveries for all of the labelled glyceryl nitrates of 97-100% (Table 5.2). None of the glyceryl nitrates was degraded in methanol during the work-up procedure.

Liquid Scintillation Spectrometry

The efficiency (E) values obtained for different degrees of quenching for the ^{3}H-toluene standard solutions in 10 ml scintillation

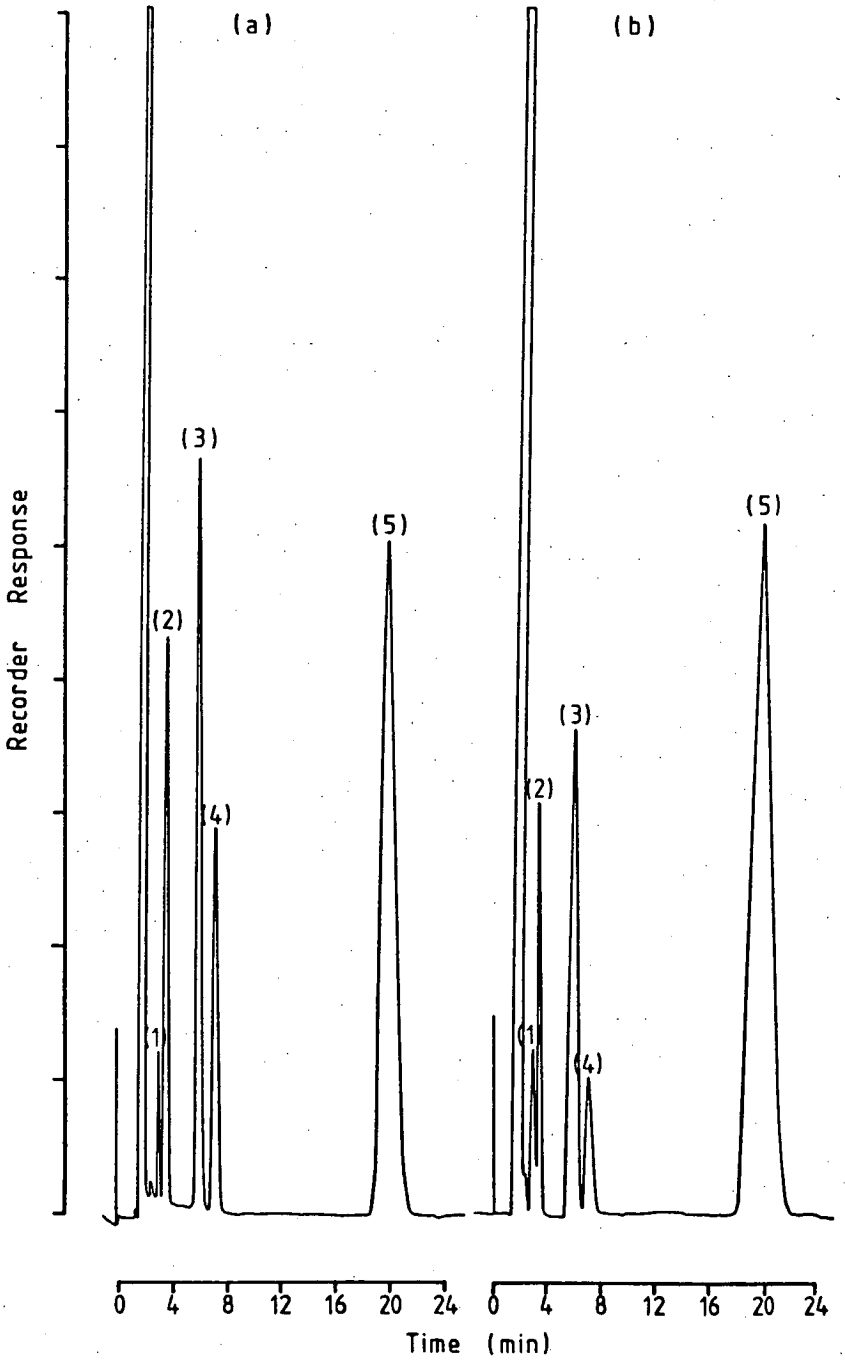


Figure 5.2 High performance liquid chromatograms of glyceryl nitrates (1, 1-GMN; 2, 2-GMN; 3, 1,3-GDN; 4, 1,2-GDN; 4, GTN) arising from (a) 20 µl and, (b) 150 µl injections of aqueous solutions using the chromatographic conditions described in Section 4.2.2.

cocktail were plotted against the ratio of counts in the channels to counts in an external standard channel in the machine (ESR). The equation describing the plot was:

$$E = -0.065\ (ESR)^2 + 0.391\ (ESR) - 0.149$$

The minimum detectability of all labelled glyceryl nitrates was set at 20 cpm above background counts.

Gas Chromatography

Figure 5.3 shows that two extractions of sheep plasma using a hexane/plasma volume ratio of 6:1 and vortexing vigorously for 1 min gave a 94% recovery of the ^{3}H-GTN. Similar results were found for sheep and human plasma. When the volume ratio was reduced to 3:1 two extractions removed only 84% of the ^{3}H-GTN (Figure 5.3). The rapid injection technique for extracting GTN from plasma with equal volumes of hexane (Yap et al., 1978) provided recovery values of ^{3}H-GTN which were both low and variable.

Evaporation of the 2.4 ml of pooled hexane extracts to 20-250 µl resulted in a further loss of GTN of 3-4%. Using the adopted method an overall recovery of about 90% was achieved for the extraction of GTN from sheep plasma.

GC tracings of blank sheep plasma and an actual sample containing GTN and the internal standard ISDN are presented in Figure 5.4 (a) and 5.4 (b) respectively. It is seen that the GTN peak is clear from any interfering peaks while ISDN is only marginally affected by a small interfering peak. The retention times of GTN and ISDN for the CC conditions used were 6.6 and 14.4 min respectively.

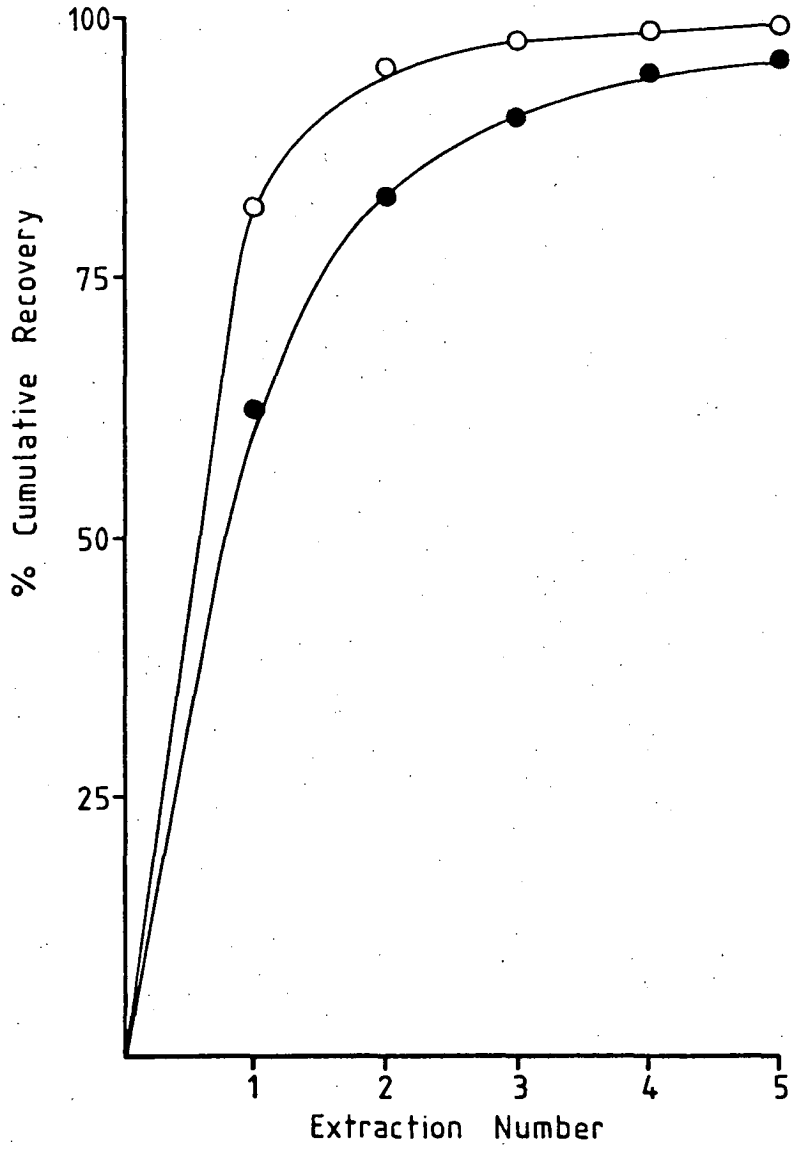


Figure 5.3 Characterization of the vortexing technique used to extract nitroglycerin from plasma with hexane (Section 4.2.4.)
(○) volume ratio of hexane : plasma of 6 : 1.
(●) volume ratio of hexane : plasma of 3 : 1.

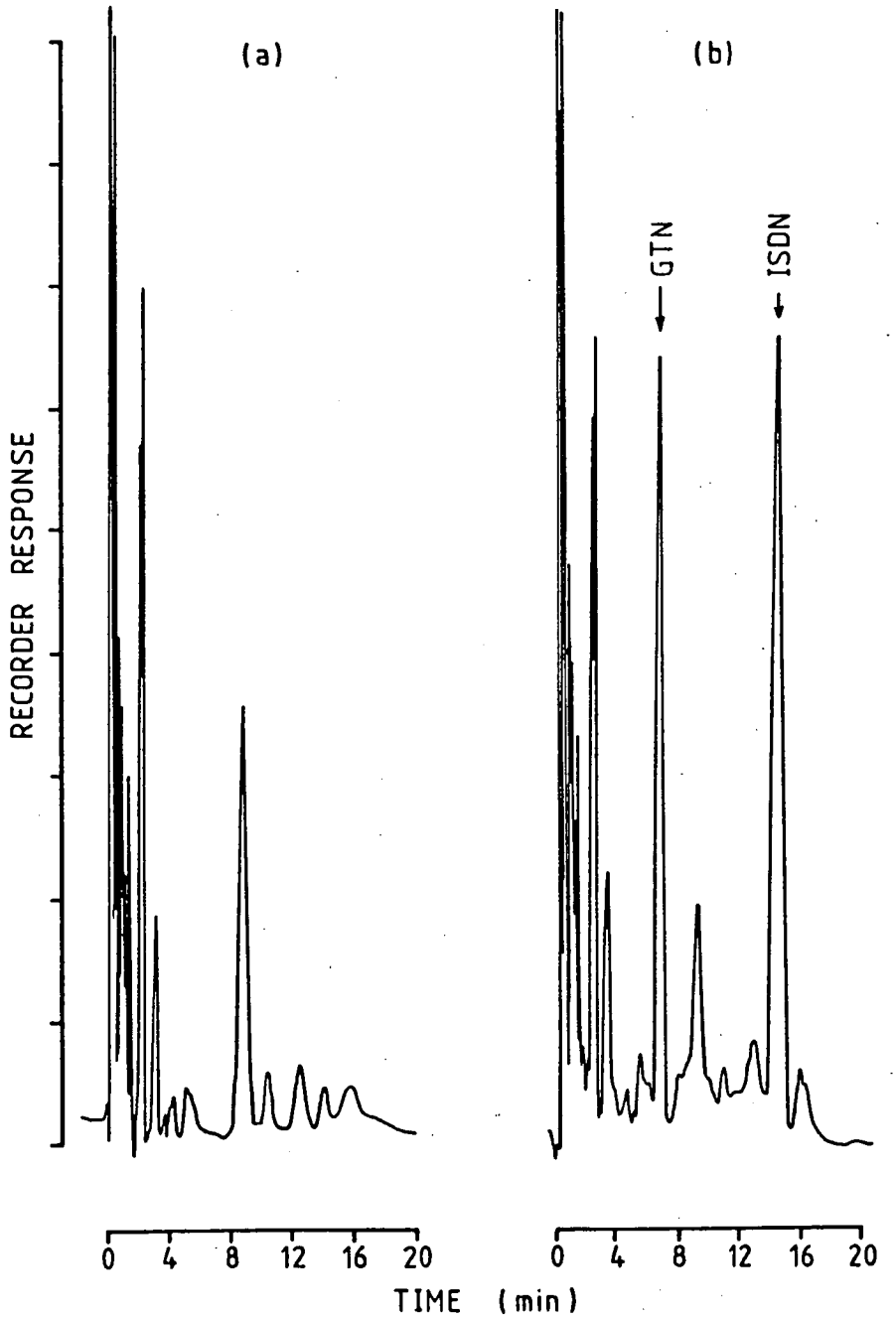


Figure 5.4 Gas chromatograms of (a) blank sheep plasma and, (b) a sample containing 15.9 ng GTN/ml plasma and the internal standard ISDN obtained using the conditions described in Section 4.2.4.

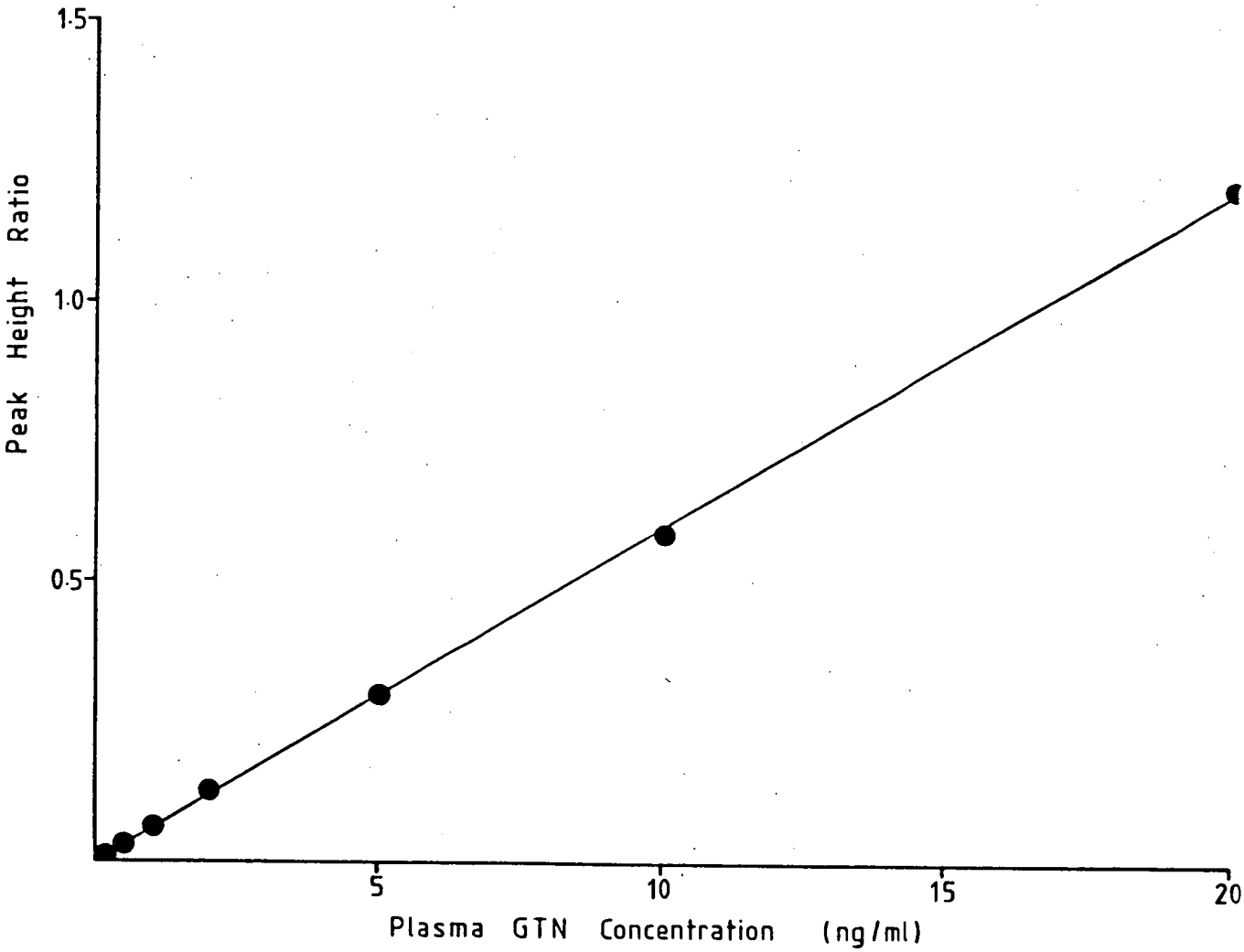


Figure 5.5 Calibration plot for the GC assay of sheep plasma GTN concentrations.

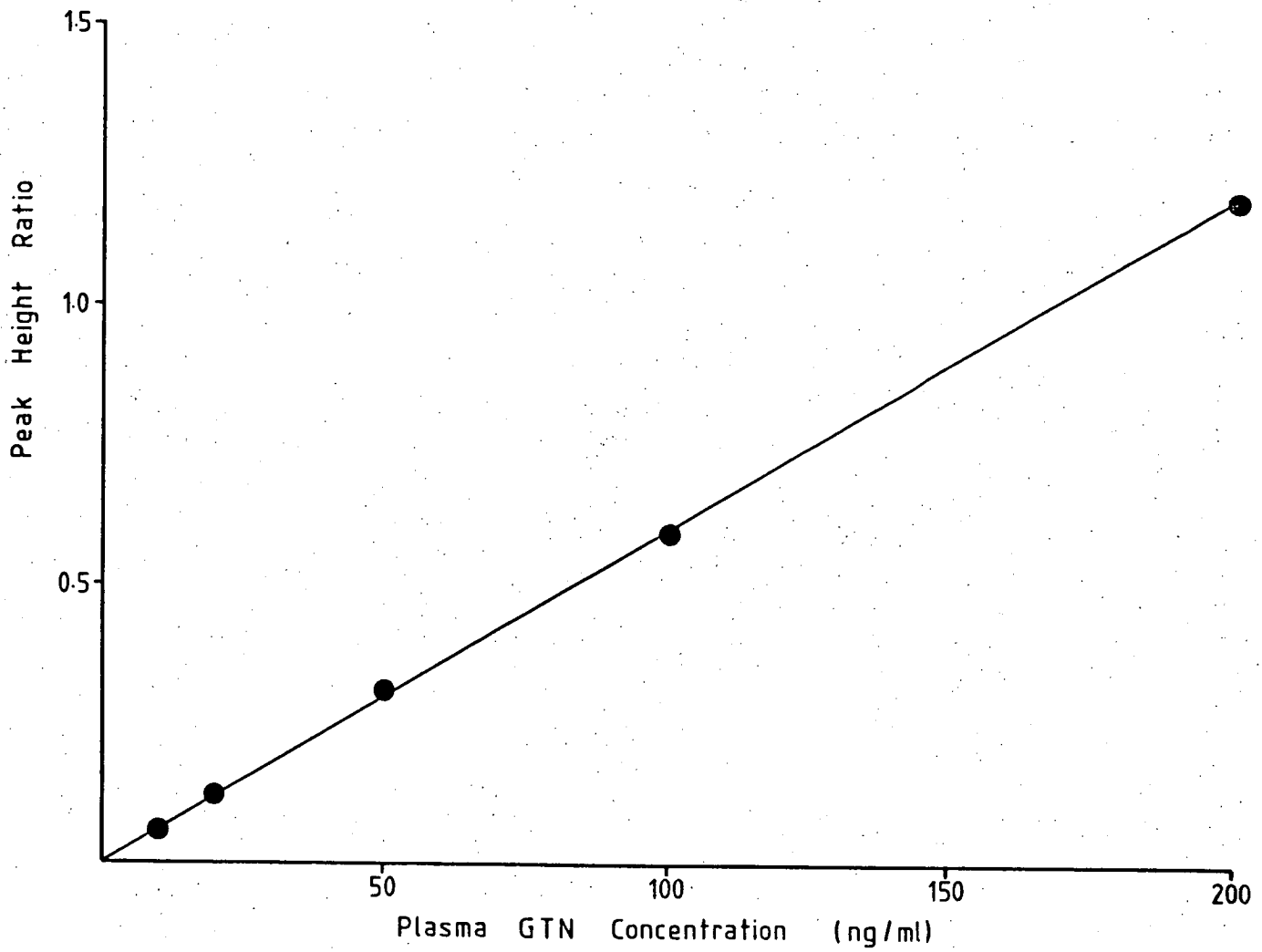


Figure 5.6 Calibration plot for the GC assay of sheep plasma GTN concentrations.

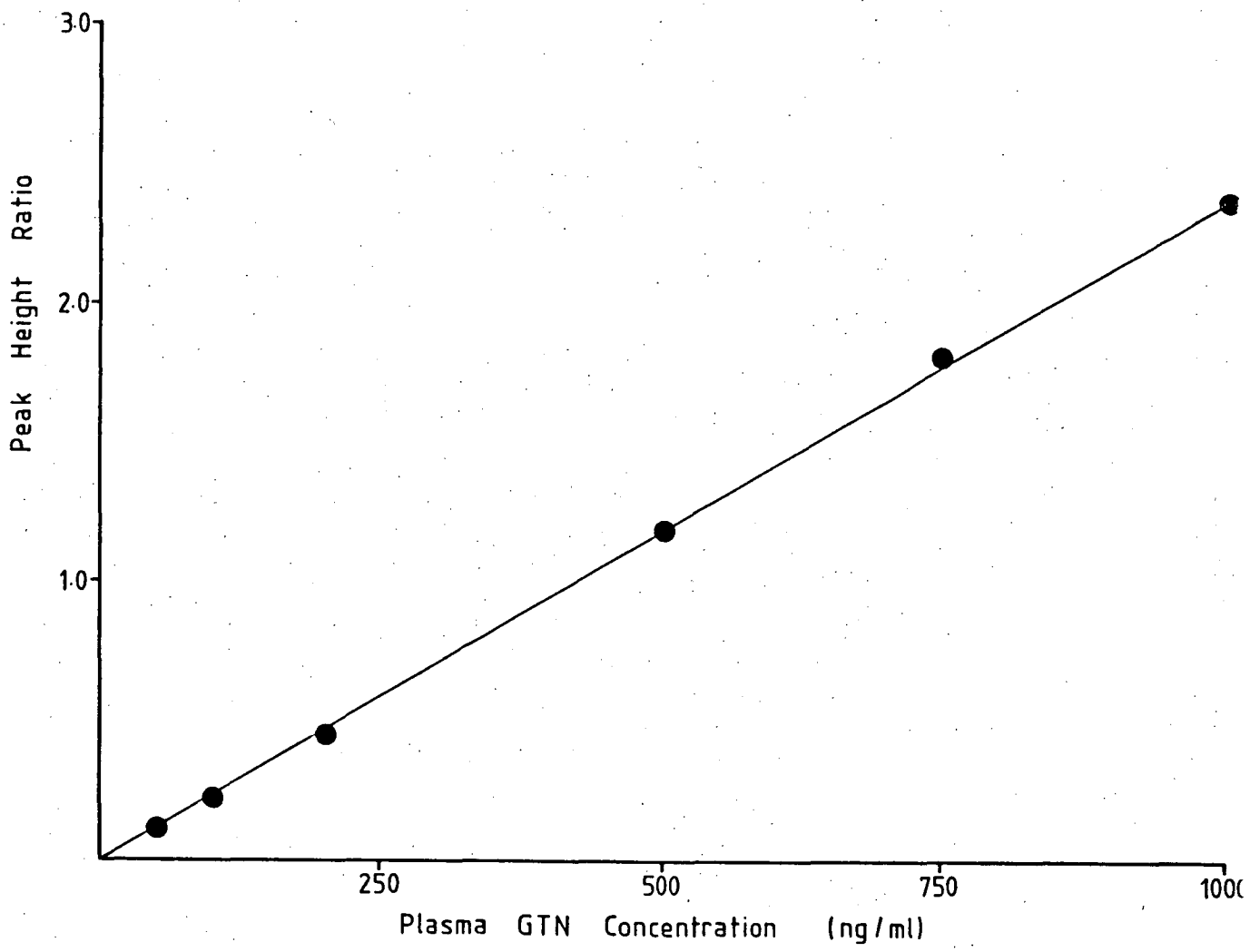


Figure 5.7 Calibration plot for the GC assay of sheep plasma GTN concentrations.

Table 5.3 Precision of the gas chromatography assay of nitroglycerin (GTN) in sheep plasma.

Amount (ng) GTN in 200 µl plasma	0.20	10.0	100.0
Mean of 5 separate determinations	0.19	9.90	101.1
Range	0.17 - 0.21	9.61 - 10.32	97.2 - 10.43
Standard Deviation	0.013	0.41	3.0
% C.V.	6.8	4.1	3.0

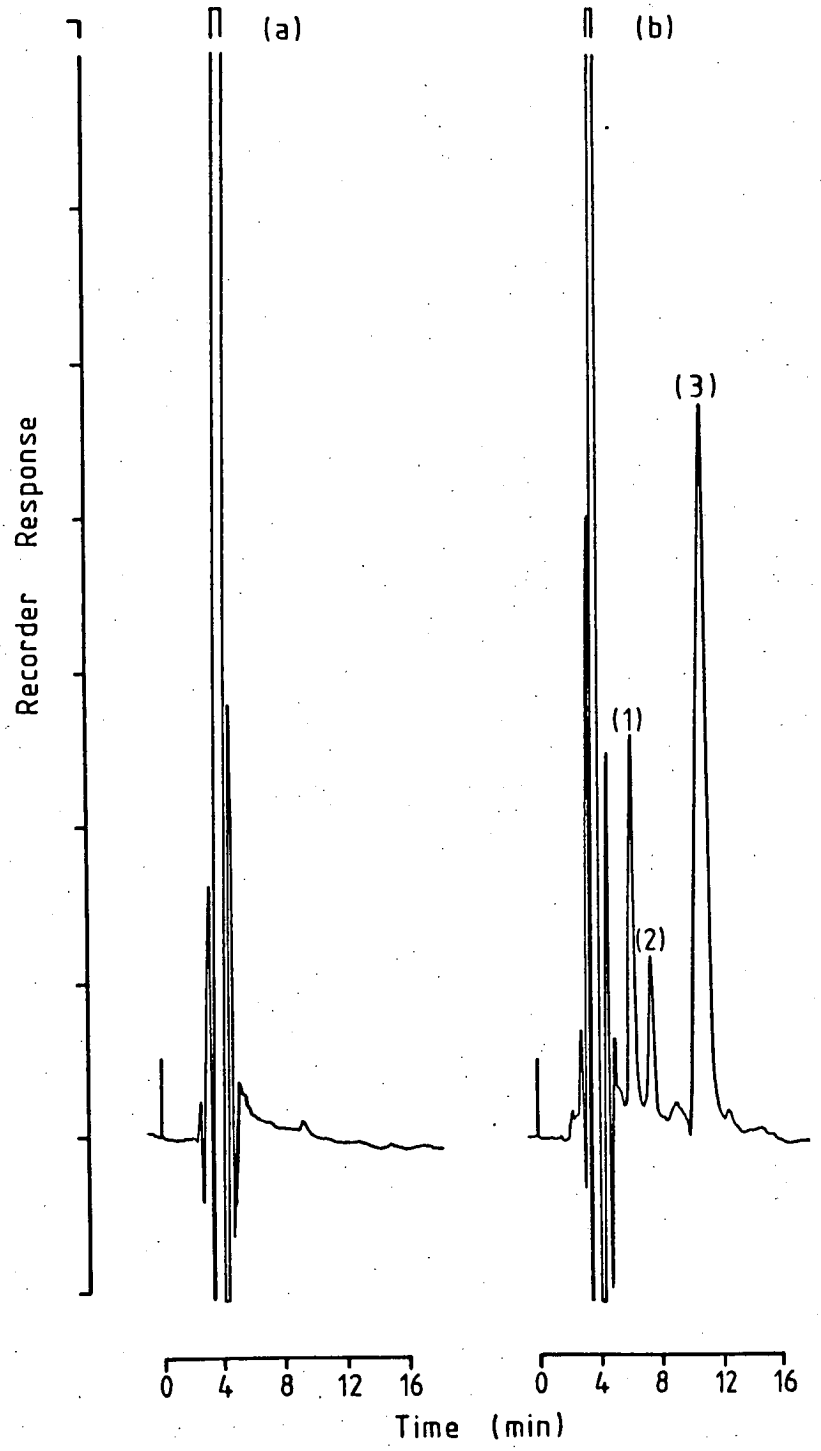


Figure 5.8 High performance liquid chromatograms of (a) blank sheep plasma and, (b) a sample of sheep plasma containing SU (1), ASA (2) and SA (3). Chromatographic conditions are described in Section 4.2.2.

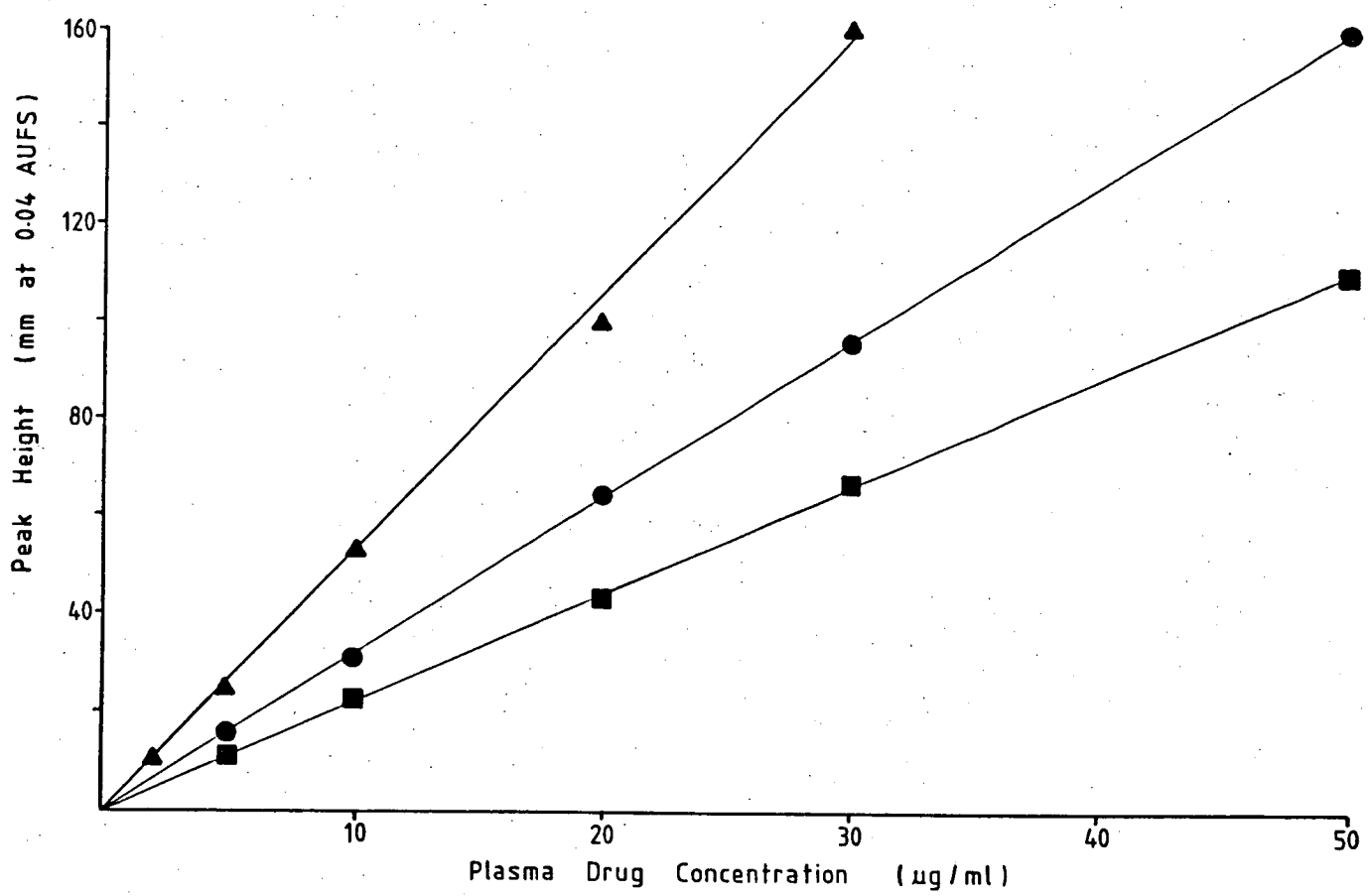


Figure 5.9 Calibration plots for the HPLC assay of sheep (and human) plasma SU (▲), ASA (●) and SA (■) concentrations.

Table 5.4 Precision of the high pressure liquid chromatography assay of aspirin (ASA) and salicylic acid (SA) in sheep plasma.

	ASA		SA	
Amount (µg) in 200 µl plasma	0.20	10.0	0.20	10.0
Mean of 5 separate determinations	0.21	10.0	0.19	9.9
Range	0.18 - 0.22	9.85 - 10.20	0.17 - 0.22	9.81 - 10.09
Standard Deviation	0.012	0.37	0.010	0.33
% C.V.	5.7	3.7	5.3	3.3

Calibration plots for three concentration ranges are presented in Figures 5.5 to 5.7. All calibration plots are linear and pass through the origin. The use of three calibration plots was made necessary by the fact that the electron capture detector in the system became saturated when more than 1 ng GTN was injected on column. Day-to-day variation in the detector response to GTN although small (<5%), made it necessary to analyze three freshly prepared plasma samples containing known amounts of GTN each day before analysis of real samples began.

The precision of the GC assay of GTN in sheep plasma is shown in Table 5.3. The coefficient of variation ranged from 6.8% to 3.0% for plasma GTN concentrations ranging from 1.0 to 500.0 ng/ml respectively. The minimum detectability of the assay was about 0.2 ng GTN/ml plasma.

5.1.2. Analysis of Salicylates

HPLC tracings of blank sheep plasma and an actual sample containing ASA, SA and SU are presented in Figure 5.8. Tests of precision and reproducibility of the HPLC assay of Rumble et al. (1981) for ASA and SA in the range 1 to 50 µg/ml sheep (or human) plasma (or buffer) showed the assay to be precise (Table 5.4) and to be reproducible with coefficients of variation in the range 5.7 to 3.7% respectively for ASA. Corresponding values for SA are 5.3 to 3.3% respectively (Table 5.4). Figure 5.9 shows typical calibration curves used to quantitate the concentrations of ASA, SA and SU in plasma.

The lower limit of detection of ASA and SA concentrations for the HPLC conditions used was about 0.1 µg/ml plasma or buffer. No tests of precision or reproducibility for plasma SU concentrations were performed.

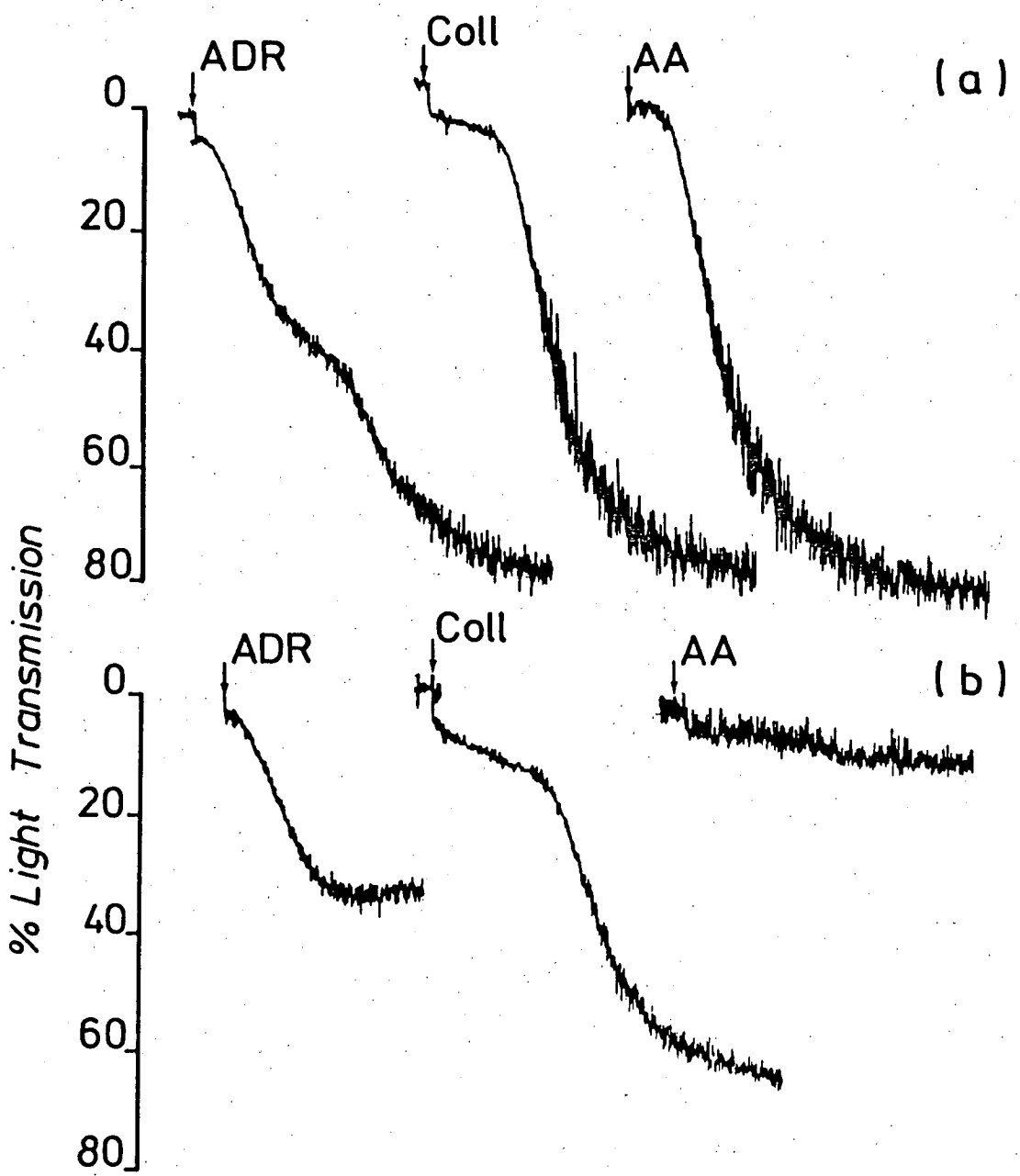


Figure 5.10 PLatelet aggregation charts from platelet-rich plasma treated with adrenalin (ADR), collagen (COLL) and arachidonic acid (AA) before commencing Astrix (a) and 3 hr after the seventh daily dose of 650 mg Astrix (b) for Subject SG.

5.1.3. Assays of Platelet Function

Platelet Aggregation

The intra- and inter-subject variability in platelet aggregation induced by adrenalin, collagen and AA is shown in Tables A1 to A3. Examples of platelet aggregation curves induced by adrenalin, collagen and arachidonic acid before and after 650 mg Astrix daily for one week are shown in Figure 5.10.

Platelet Malondialdehyde Production

Platelet counts determined after resuspending the platelet button in Tris buffer showed that 87% of the platelets were recovered from the washing process. The molar absorbance of MDA in saline was found to be 1.56×10^5. Using platelet rich plasma from the same blood sample for 10 separate determinations for NEM and 7 for AA, the overall coefficient of variation was 8%.

5.1.4. Plasma Protein Binding

The glyceryl nitrates and salicylates were not sorbed by the plastic ultrafiltration devices or membranes (Table 5.5) when tested using drug concentrations which fall within the range of plasma concentrations commonly encountered in the experimental work described in other sections of this thesis.

During experiments involving the infusion of aspirin into sheep, it was observed that the plasma albumin concentrations began to fall from control values (when the sheep was conscious) during the anaesthetic and surgical procedures necessary to implant the catheters and flow-probes, and then settled at about 60% of control values during

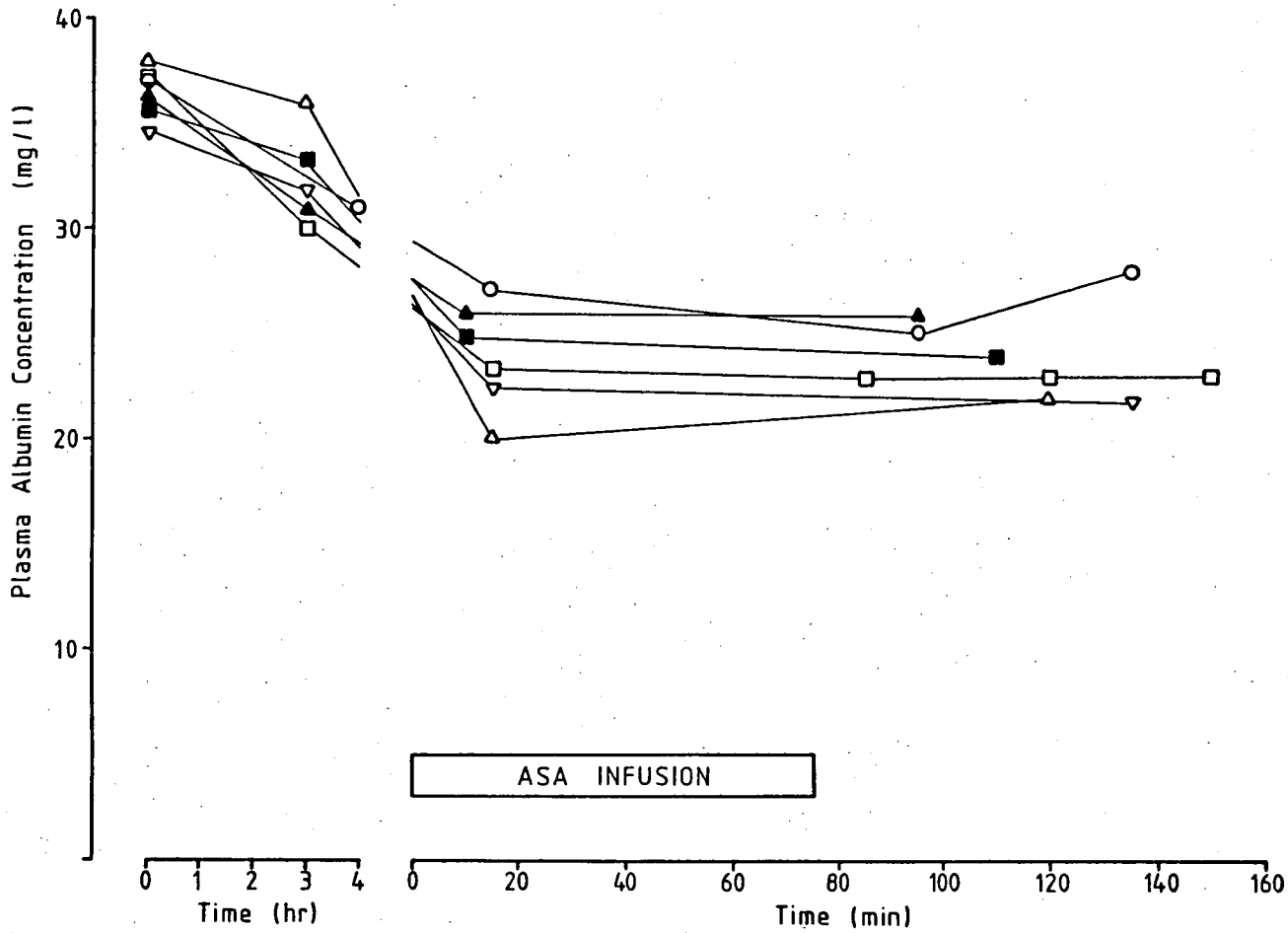


Figure 5.11 Plasma albumin concentrations of sheep undergoing anaesthesia and surgery in preparation for intravenous infusions of ASA. (Sheep 25 (△); Sheep 26 (▲); Sheep 27 (□); Sheep 28 (▽); Sheep 29 (○); Sheep 30 (■).

Table 5.5 Recoveries of drugs in control experiments to determine losses to plastic ultrafiltration apparatus and membranes. (Mean ± S.E. (n = 3)).

Drug	Drug Concentration (µg/ml)	% Recovery
GTN	0.005	99.0 ± 1.0
1, 3-GDN	0.011	99.8 ± 0.09
1, 2-GDN	0.012	100.0 ± 0.02
ASA	5.0	100.2 ± 0.18
SA	10.1	99.9 ± 0.08

Table 5.6 In vitro plasma protein binding of aspirin (ASA) in the plasma of untreated Sheep Nos. 27[a] and 29[b] and the effect of salicylic acid (SA), potassium fluoride (KF), Nembutal and plasma dilution on the protein binding.

Treatment	ASA (µg/ml)	SA (µg/ml)	Fraction Unbound a	Fraction Unbound b
Plasma	10	-	0.36	0.34
	10	50	0.48	0.49
	50	-	0.38	0.36
	50	50	0.49	0.50
	-	10	0.15	0.18
	-	50	0.22	0.21
Dilute Plasma 1:1	10	-	0.48	0.50
	10	50	0.70	0.71
	50	50	0.69	0.73
	-	50	0.27	0.33
Plasma + KF (2.5 mg/ml)	10	-	0.35	0.36
Plasma + Nembutal (10 µg/ml)	10	-	0.37	0.36
Dilute Plasma + KF + Nembutal	10	-	0.49	0.50
	10	10	0.53	0.51
	10	50	0.68	0.66

the infusion of drug and post-infusion drug sampling times (Figure 5.11) In vitro experiments demonstrated that plasma dilution (which simulates reduced plasma albumin concentrations) reduced the binding of both ASA and SA when compared to undiluted plasma (Table 5.6). Moreover, ASA binding was reduced in the presence of SA in undiluted plasma but to a much larger degree in diluted plasma (Table 5.6). Potassium fluoride and Nembutal (pentobarbitone) had negligible effect on the binding of ASA in undiluted or diluted plasma.

The binding of GTN to plasma proteins was constant over a wide GTN concentration range for human and sheep plasma (Tables 5.7 and 5.8).

5.2 IN VITRO DISPOSITION OF NITROGLYCERIN IN BLOOD COMPONENTS

5.2.1. Metabolism of Nitroglycerin in Erythrocyte Suspensions

Figure 5.12 shows that GTN is degraded to the dinitrate metabolites when it is incubated with resuspended human erythrocytes. Detection of the GMN metabolites is prevented on the HPLC chromatogram by interfering endogenous substances with the same retention time as the GMNs. Although the concentration of GTN (100 µg/ml) used to obtain this chromatogram was very much greater than therapeutic concentrations in man, similar patterns of degradation were found for initial GTN concentrations of 600, 50, 10, 2 and 0.8 ng/ml blood or suspension using the tritiated GTN. Figure 5.13 shows the time course of GTN disappearance from and 1,2-GDN and 1,3-GDN and GMN evolution into the buffer of the suspension for an initial GTN concentration of 10 ng/ml suspension. Similar results were obtained for both human and sheep erythrocyte suspensions.

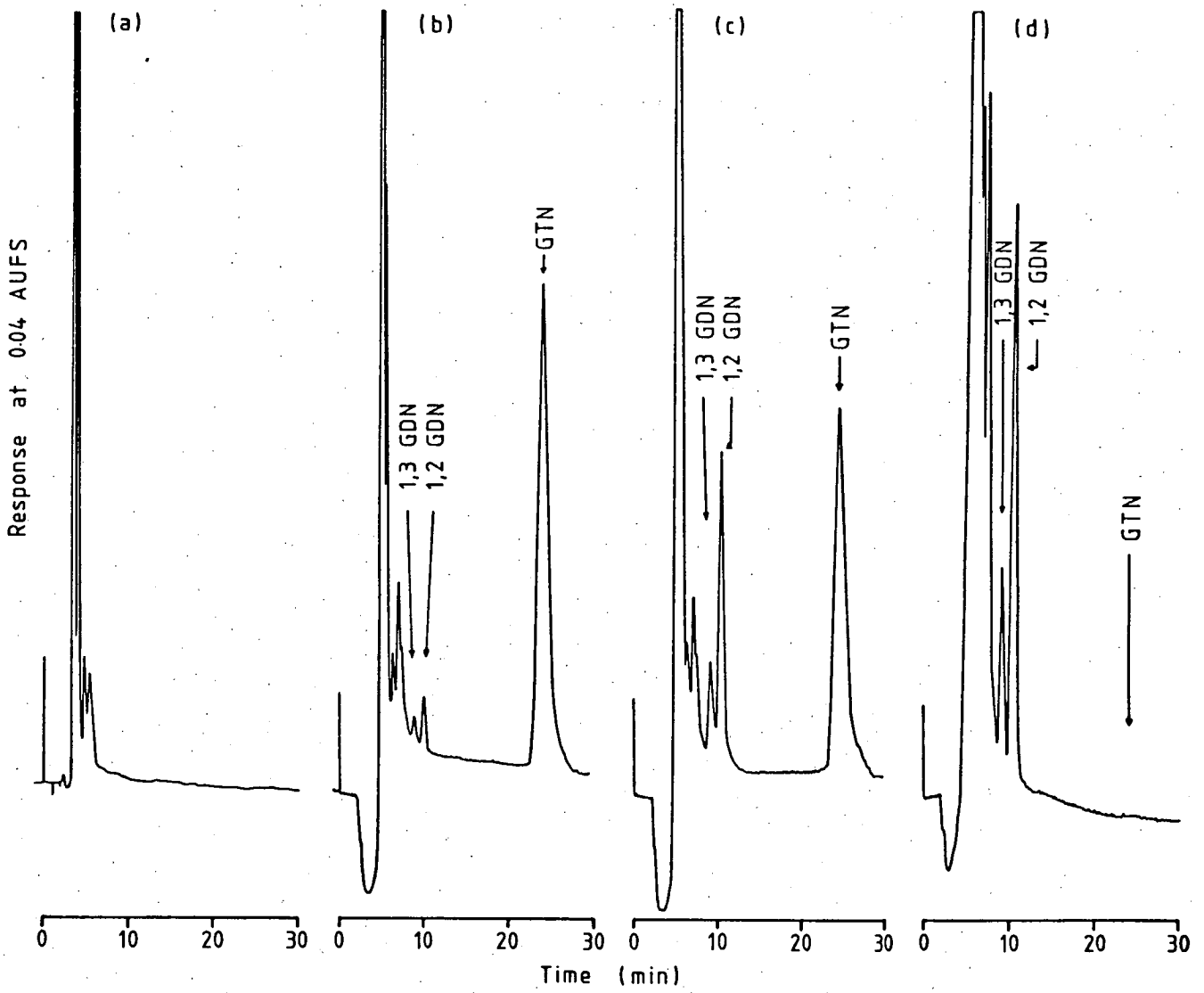


Figure 5.12 HPLC tracings showing GTN (initial concentration 100 µg/ml) metabolism in resuspended human erythrocytes at 37°C. (a) blank suspension; (b) immediately after adding GTN; (c) after 10 min incubation; (d) after 2 hr incubation. HPLC conditions are described in Section 4.2.2.

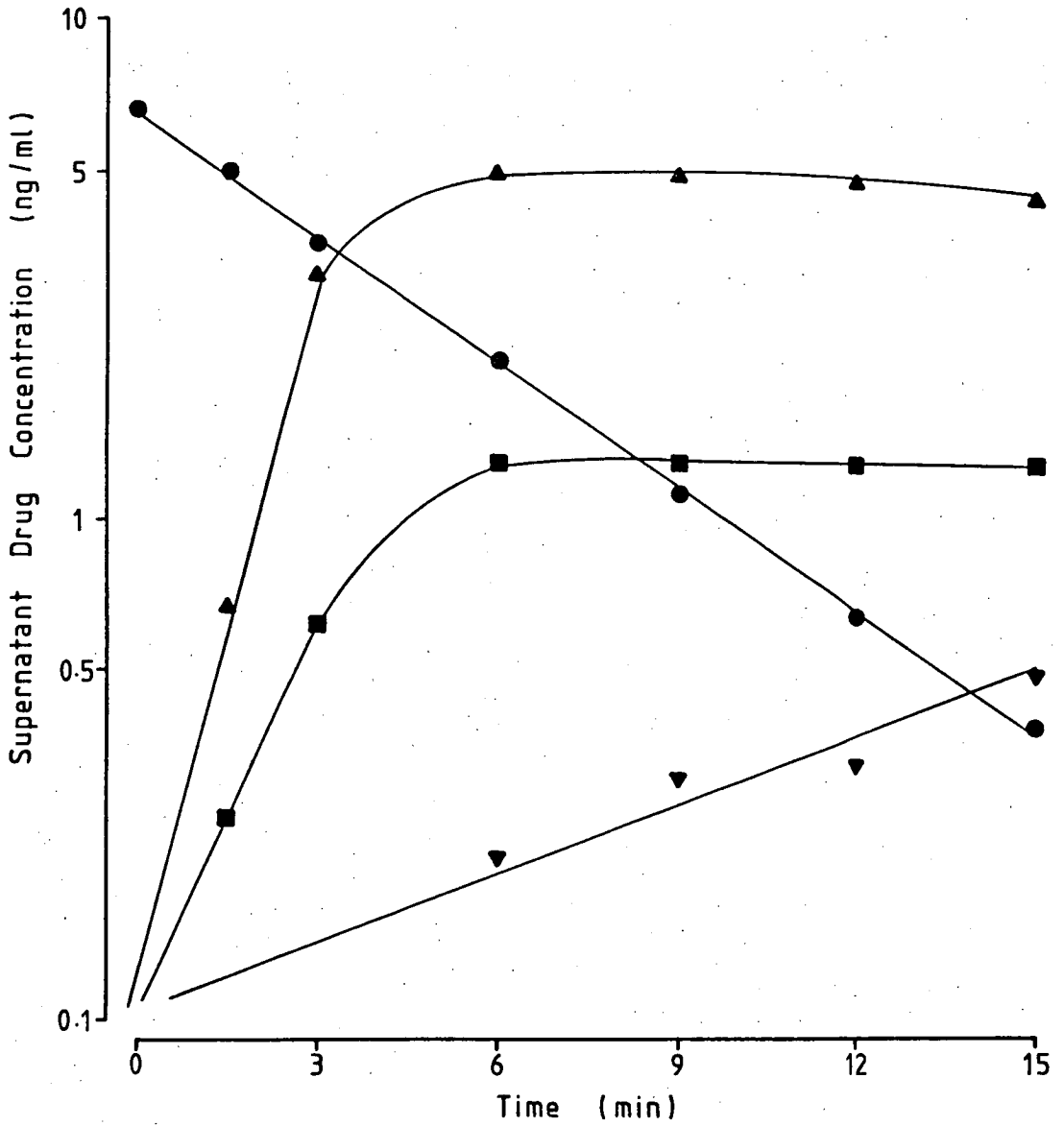


Figure 5.13 Time course of GTN metabolism in resuspended human erythrocytes at 37°C. (●) GTN; (▲) 1,2-GDN; (■) 1,3-GDN; (▼) GMNs. Erythrocytes were collected from Subject B and the initial GTN concentration was 10 ng/ml suspension. Glyceryl nitrate concentrations were measured in the supernatant buffer of the suspension.

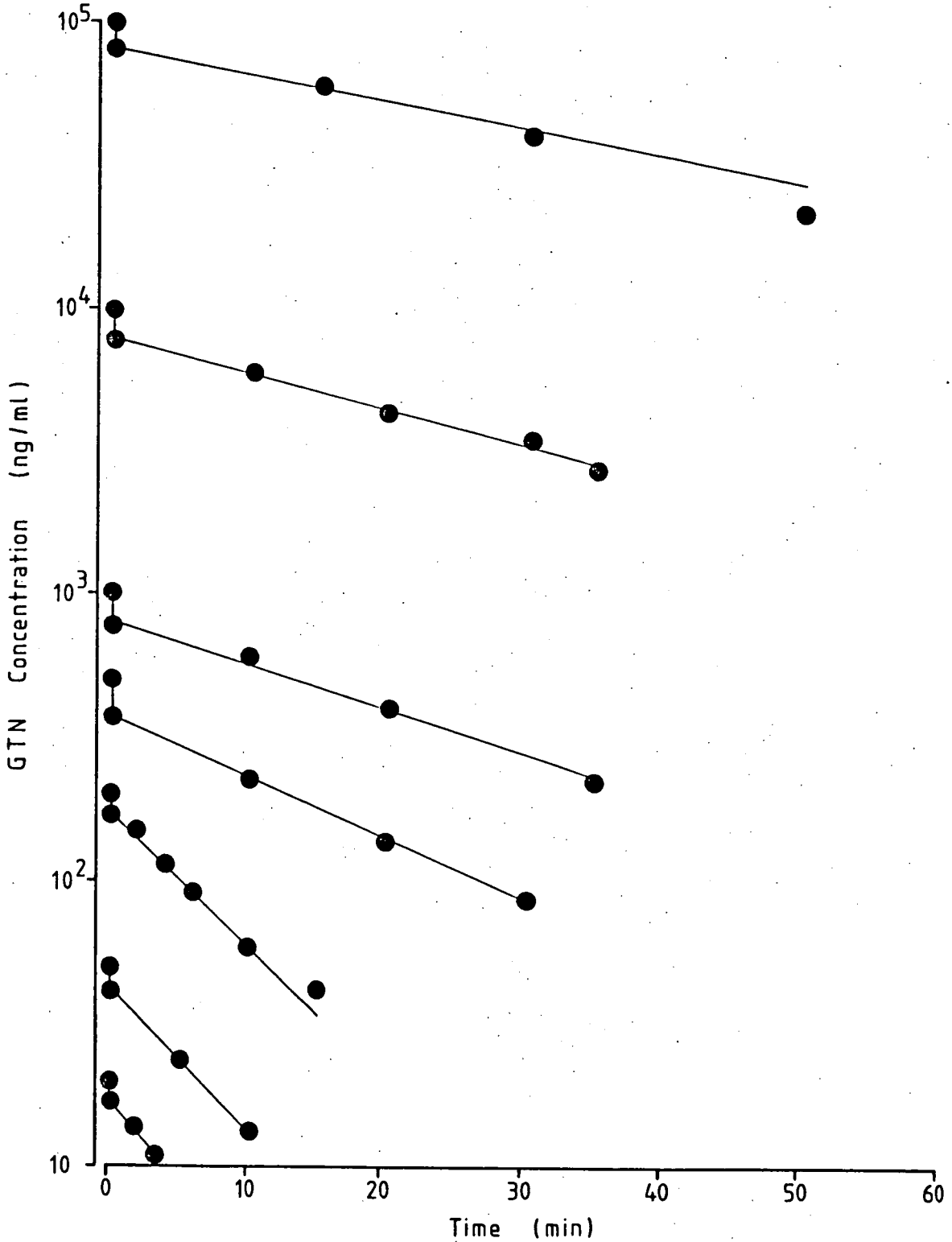


Figure 5.14 Influence of the initial GTN concentration on the rate of GTN metabolism in resuspended human erythrocytes of Subject A.

Table 5.7 Half-lives of metabolism in blood and resuspended erythrocytes, apparent erythrocyte/buffer and erythrocyte/plasma partition coefficients and bound fraction to plasma proteins of nitroglycerin (GTN), 1, 2-dinitroglycerin (1, 2-GDN) and 1, 3-dinitroglycerin (1, 3-GDN). (Mean ± S.E. (n = 3)).

	Drug concentration (ng/ml)	Half-life of metabolism (min)			Apparent erythrocyte/ buffer partition coefficient	Apparent erythrocyte/ plasma partition coefficient	Fraction bound to plasma proteins
		Plasma	Blood	Resuspended erythrocytes			
GTN	600	-	16.6 ± 0.40	17.0 ± 0.51	2.14 ± 0.04	0.59 ± 0.016	0.63 ± 0.013
	50	52.7 ± 2.1	6.1 ± 0.18	6.0 ± 0.20	2.09 ± 0.04	0.57 ± 0.014	0.61 ± 0.011
	10	51.3 ± 1.9	3.7 ± 0.23	3.6 ± 0.19	2.10 ± 0.05	0.61 ± 0.011	0.64 ± 0.012
	2	-	3.0 ± 0.15	3.1 ± 0.18	2.09 ± 0.05	0.58 ± 0.013	0.61 ± 0.013
	0.8	-	2.7 ± 0.17	2.9 ± 0.13	2.06 ± 0.08	0.58 ± 0.011	0.61 ± 0.013
1,2-GDN	23.3	-	35.2 ± 1.10	35.4 ± 0.99	2.04 ± 0.05	0.84 ± 0.024	0.23 ± 0.016
1,3-GDN	21	-	48.1 ± 0.95	49.0 ± 1.30	2.11 ± 0.06	0.88 ± 0.018	0.11 ± 0.029

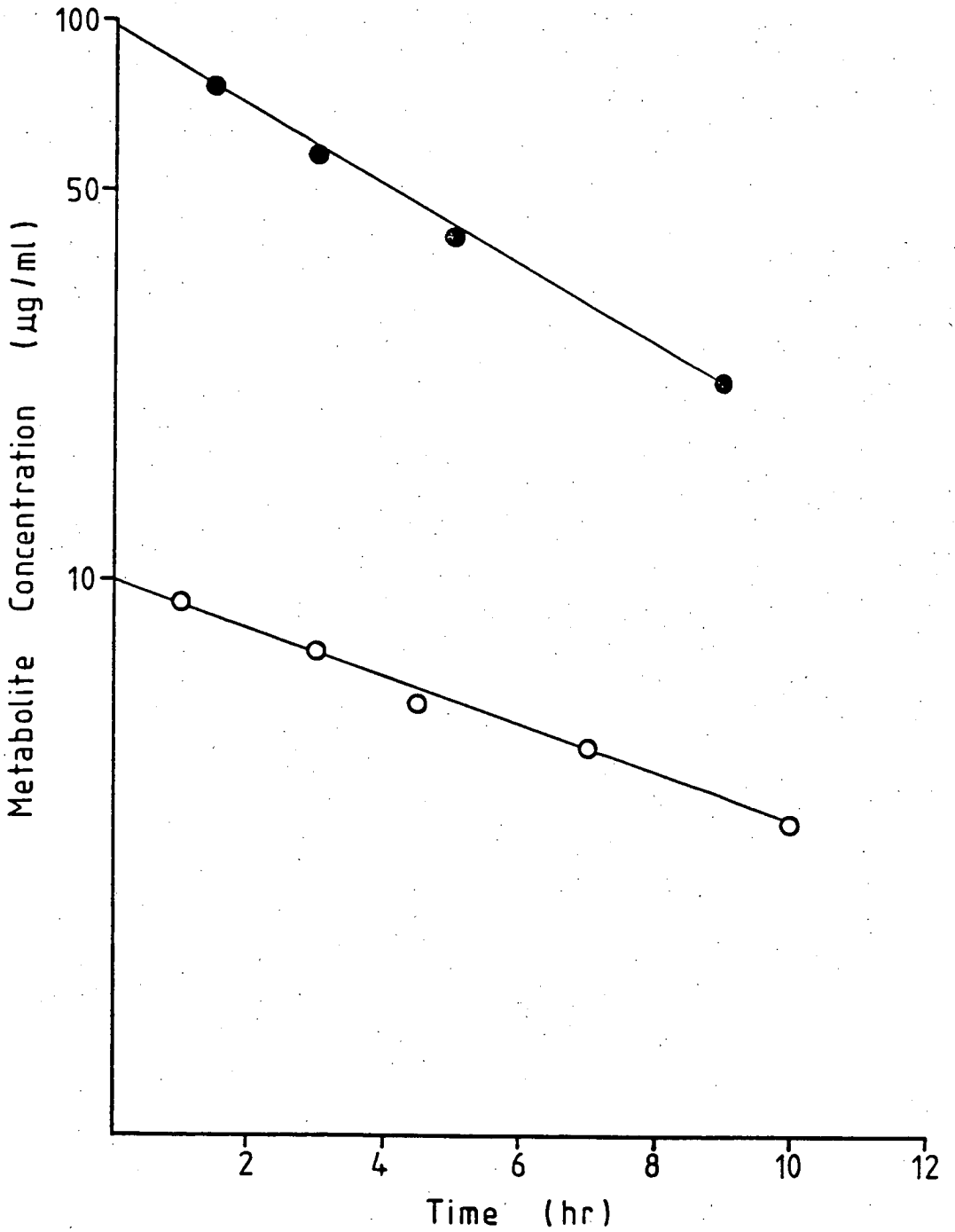


Figure 5.15 Disappearance of 1,2-GDN (●) and 1,3-GDN (○) from solutions incubated with resuspended human erythrocytes (Subject A) at 37°C.

Table 5.8 Half-lives of metabolism in sheep blood, resuspended erythrocytes and plasma, apparent erythrocyte/buffer and erythrocyte/plasma partition coefficients and bound fraction to plasma proteins of nitroglycerin (GTN), 1, 2-dinitroglycerin (1, 2-GDN) and 1, 3-dinitroglycerin (1, 3-GDN). (Mean ± S.E. (n = 3)).

	Drug concentration (ng/ml)	Half-life of metabolism (min)			Apparent Erythrocyte/ Buffer Partition Coefficient	Apparent Erythrocyte/ Plasma Partition Coefficient	Fraction Bound to Plasma Proteins
		Plasma	Blood	Resuspended Erythrocytes			
GTN	50	51.3 ± 1.7	6.3 ± 0.31	6.3 ± 0.28	2.10 ± 0.03	0.60 ± 0.041	0.62 ± 0.011
	10	50.7 ± 1.3	3.5 ± 0.11	3.6 ± 0.41	2.17 ± 0.01	0.60 ± 0.013	0.60 ± 0.008
1,2-GDN	23.6	-	36.8 ± 1.1	36.7 ± 0.58	2.09 ± 0.04	0.82 ± 0.017	0.23 ± 0.014
1,3-GDN	20.7	-	49.3 ± 1.1	49.9 ± 0.92	2.09 ± 0.04	0.88 ± 0.017	0.12 ± 0.011

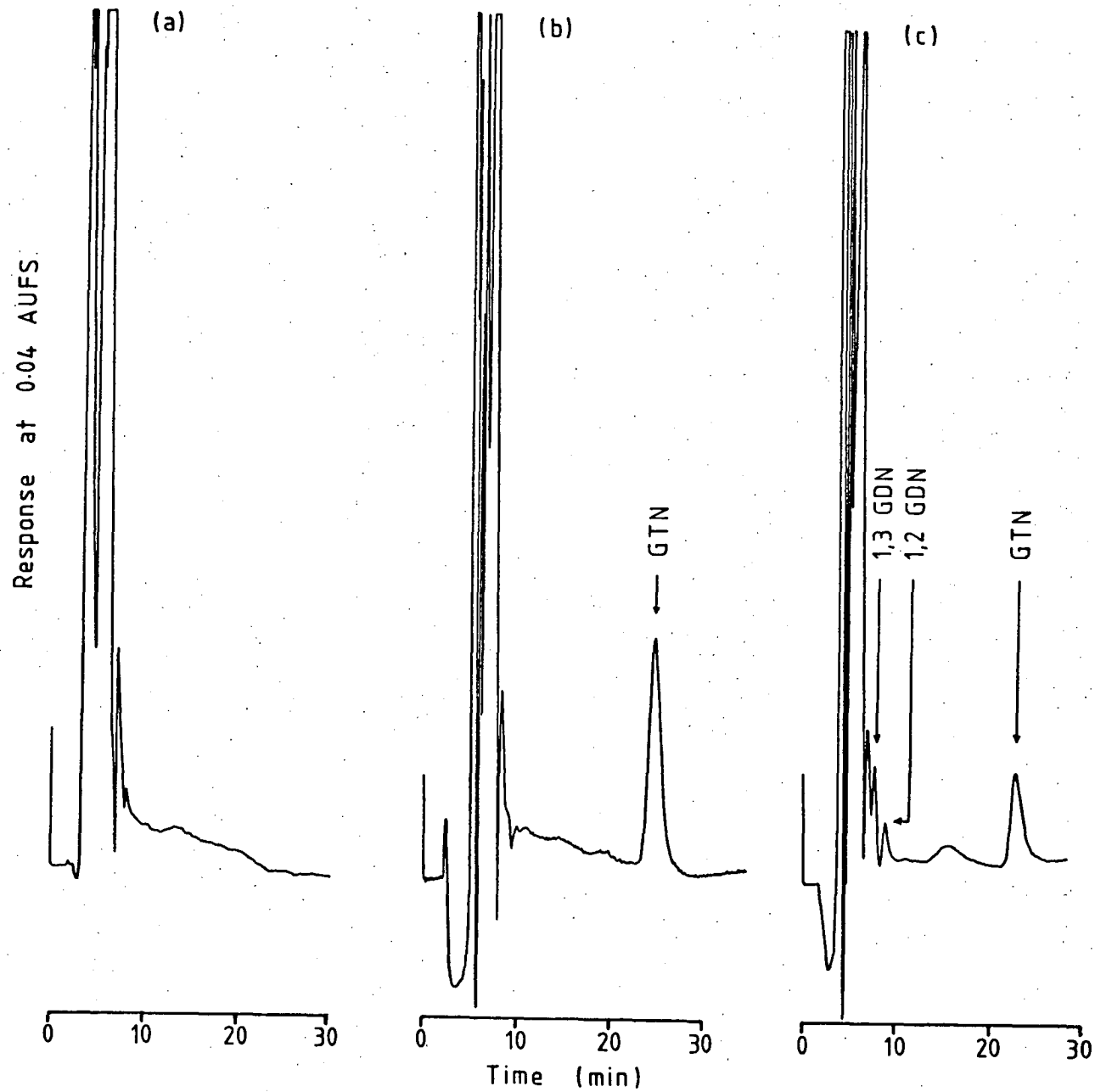


Figure 5.16 HPLC tracings showing GTN (initial concentration 10 µg/ml) metabolism in human plasma at 37°C. (a) blank plasma; (b) immediately after adding GTN; (c) after 2 hr incubation. HPLC conditions are described in Section 4.2.2.

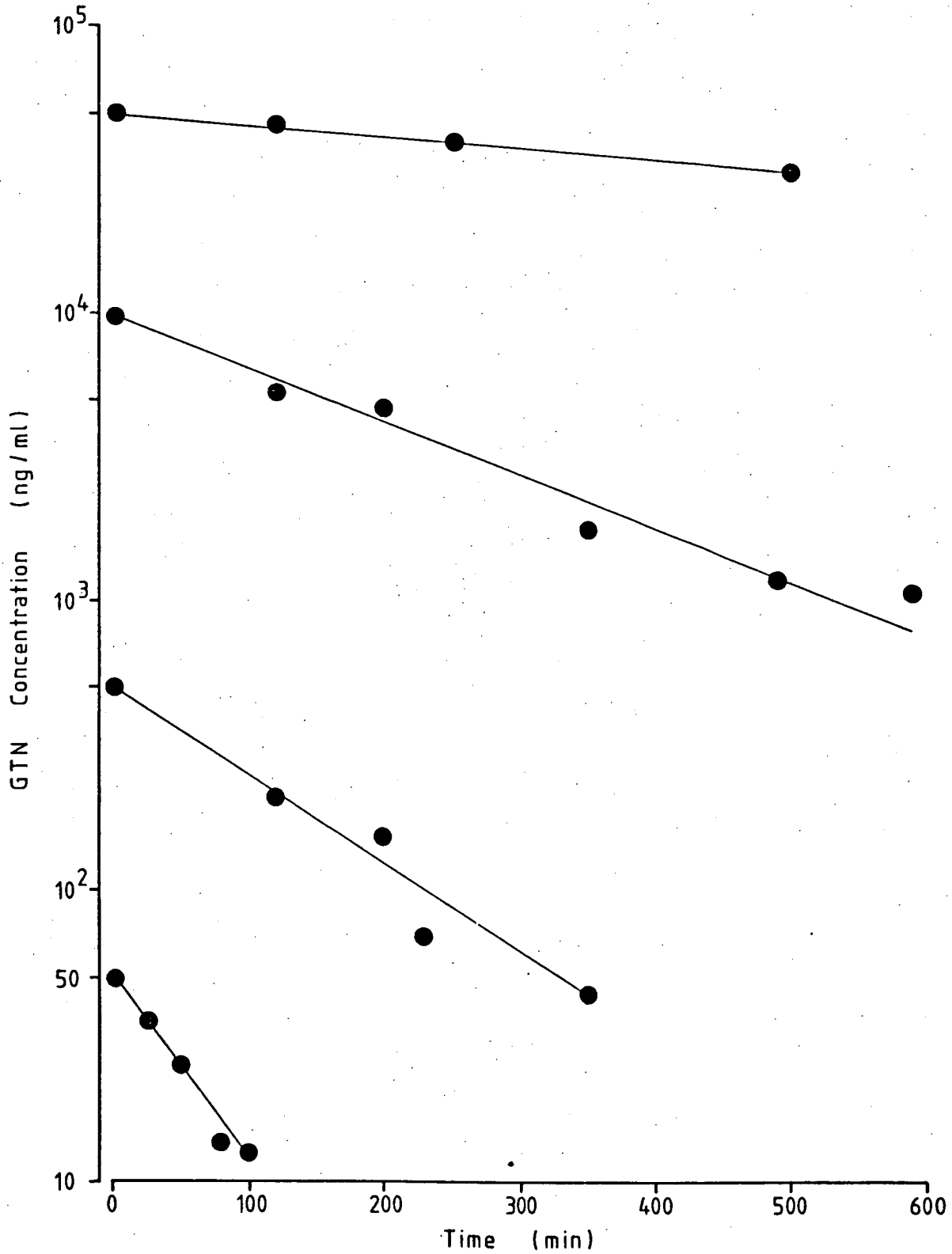


Figure 5.17 Influence of the initial GTN concentration on the rate of metabolism in human plasma (Subject A).

Table 5.9

The effect of added 1, 2- dinitroglycerin (1, 2- GDN) and 1, 3- dinitroglycerin (1, 3- GDN) on the half-life of metabolism of nitroglycerin (GTN) by resuspended erythrocytes. (Mean ± SE, n=3)

GTN Concentration (ng/ml)	1, 2- GDN Concentration (ng/ml)	1, 3- GDN Concentration (ng/ml)	Half-life of GTN metabolism (min)
9.8	0	0	3.4 ± 0.26
10.0	50	0	6.3 ± 0.21
9.8	600	0	15.9 ± 0.39
9.9	0	50	6.5 ± 0.24
10.0	0	600	16.6 ± 0.41

Table 5.10 The effect of initial concentration on the disappearance of 1, 2- and 1, 3- dinitroglycerin from erythrocyte suspensions at 37°C. (Mean ± SE, n=3)

Initial concentration (µg/ml)	Apparent half-life of disappearance (hrs)	
	1, 2- GDN	1, 3- GDN
60	3.4 ± 0.21	5.9 ± 0.26
10	3.0 ± 0.20	5.1 ± 0.26
1	2.0 ± 0.26	3.6 ± 0.18

Figure 5.14 shows that the time course of GTN disappearance from human erythrocyte suspensions is concentration dependent; the rate of GTN disappearance increases at the lower initial GTN concentrations. In the second study which was designed to quantitate the rapid initial uptake of GTN, similar concentration-dependent losses of GTN due to metabolism were observed as shown in Figure 5.14. The results of the second experiment are presented in Table 5.7 for resuspended human erythrocytes and Table 5.8 for resuspended sheep erythrocytes.

When 1,2-GDN and 1,3-GDN were added to human erythrocyte suspensions containing GTN at a concentration of about 10 ng/ml, the rate of GTN metabolism decreased as the added GDN concentrations increased (Table 5.9). GDNs were also metabolized in resuspended erythrocytes (Fig. 5.15). Table 5.10 shows that the apparent half-lives of the GDN metabolites were also concentration dependent.

5.2.2. Metabolism of Nitroglycerin in Plasma

GTN was metabolized by both human and sheep plasma to form the GDNs. The metabolism of GTN differed between plasma and resuspended erythrocytes with the 1,3-GDN being the major metabolite formed in plasma (Fig. 5.16) and 1,2-GDN, the major metabolite formed in erythrocyte suspensions (Fig. 5.12).

Tables 5.7 and 5.8 show the half-lives of GTN in human and sheep plasma, respectively, for GTN concentrations of 50 and 10 ng/ml. The half-lives at these GTN concentrations were 50-52 min for human and sheep plasma. When compared to the half-lives of GTN in blood or resuspended erythrocytes it can be seen that degradation of GTN in plasma is very slow (Tables 5.7 and 5.8).

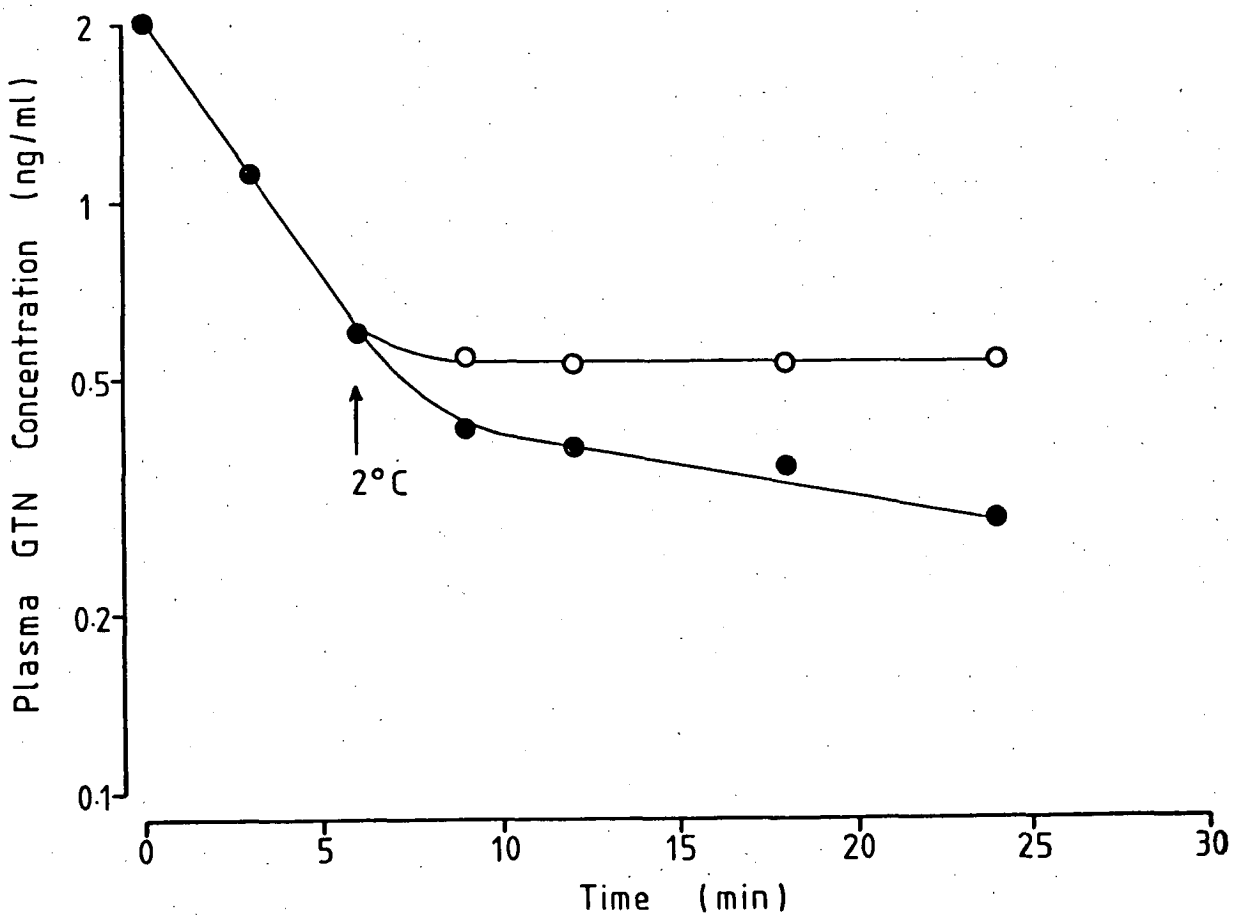


Figure 5.18 The effect of iodoacetamide on the rate of metabolism of GTN in human blood (Subject A). GTN (1.5 ng/ml) was incubated with blood at 37°C for 6 min after which the contents of the incubation vial were split and placed in pre-cooled tubes containing iodoacetamide (4mM), (○) or buffer (●).

The metabolism of GTN in (human) plasma is concentration dependent; the very large GTN concentrations being associated with the longer apparent half-lives (Figure 5.17).

5.2.3. Distribution of Glyceryl Nitrates in Blood and Erythrocyte Suspensions

Apparent human or sheep erythrocyte/buffer partition coefficients were about 2.1 for GTN, 1,3-GDN and 1,2-GDN; however, the presence of plasma considerably reduced the initial uptake of the three drugs by erythrocytes so that the apparent erythrocyte/plasma partition coefficient for GTN was about 0.60 while that of the GDNs was about 0.85 (Tables 5.7 and 5.8). The altered distribution in blood is reflected by the relative degree of plasma protein binding of the three drugs (Tables 5.7 and 5.8).

5.2.4. Inhibition of Nitroglycerin Metabolism by Iodoacetamide

Figure 5.18 shows the time course of GTN in the plasma of blood samples containing iodoacetamide. When blood containing GTN was removed from incubation at 37°C and placed in a chilled glass test tube containing iodoacetamide at a final concentration of 4mM, the degradation of GTN was stopped almost immediately (Figure 5.18). In contrast, the degradation of GTN in the absence of iodoacetamide continued at 2°C but at a much slower rate than observed at 37°C (Figure 5.18). Similar results were also obtained for sheep blood.

5.3. IN VITRO METABOLISM OF NITROGLYCERIN IN SHEEP TISSUES

Figures 5.19 to 5.22 show the extent of GTN loss from tissue homogenates when incubated at 37°C for various times. Substantial

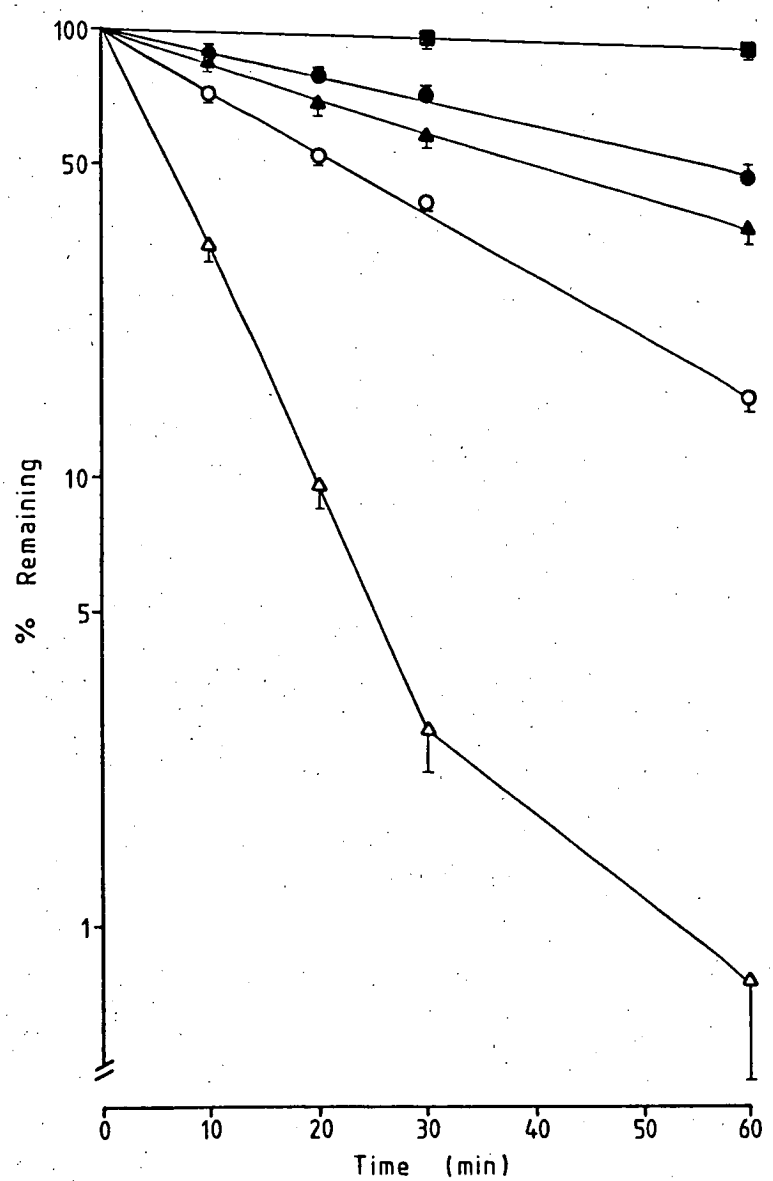


Figure 5.19 Metabolism of GTN (50 ng/ml) in suspensions of sheep liver (▲ △) and lung (● ○) tissue, with (closed symbols) and without (open symbols) added GDNs (each 100 ng/ml). Control incubations (■ □). Each point represents the mean ± se of the results for three animals.

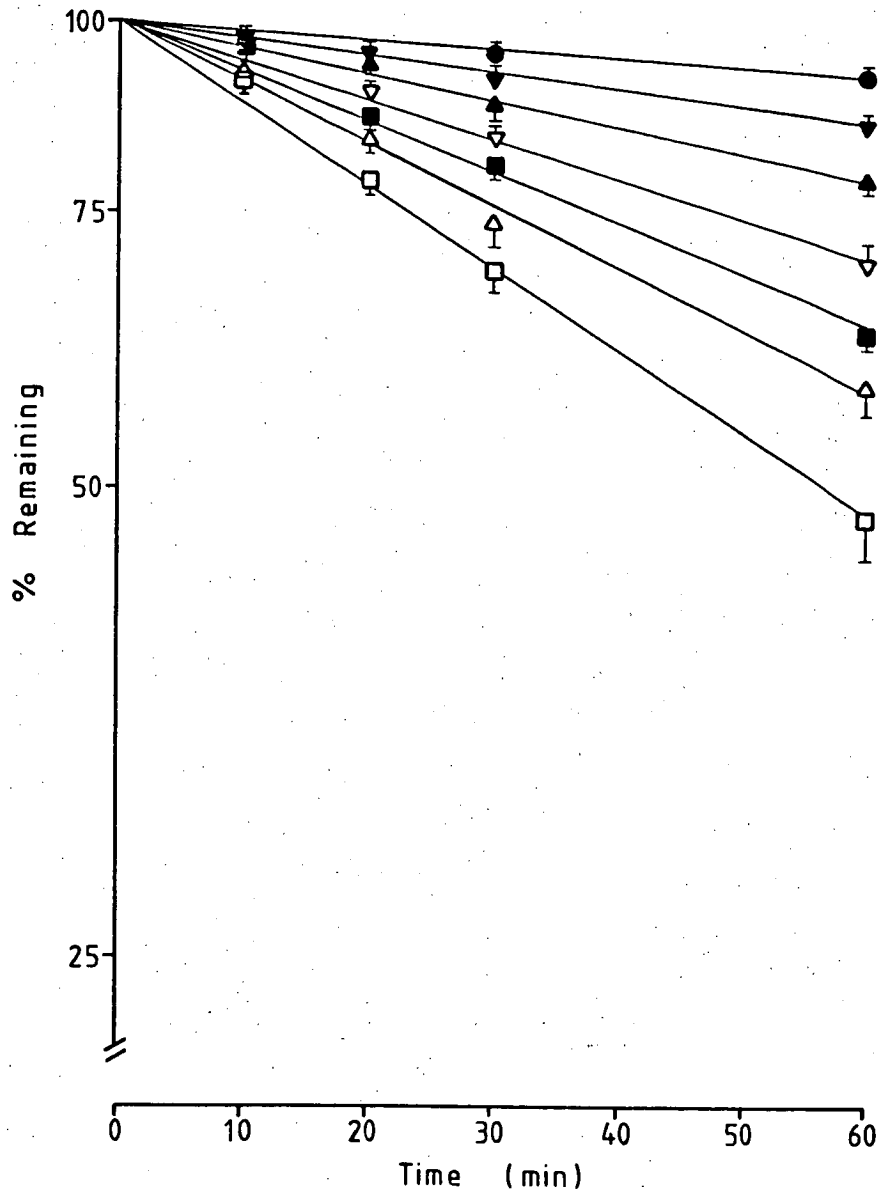


Figure 5.20 Metabolism of GTN (50 ng/ml) in suspensions of sheep leg muscle (■ □), aorta (▲ △) and vena cava (▼ ▽) tissue with (closed symbols) and without (open symbols) added GDNs (each 100 ng/ml). Control incubations (● ○). Each point represents the mean ± se of the results for three animals.

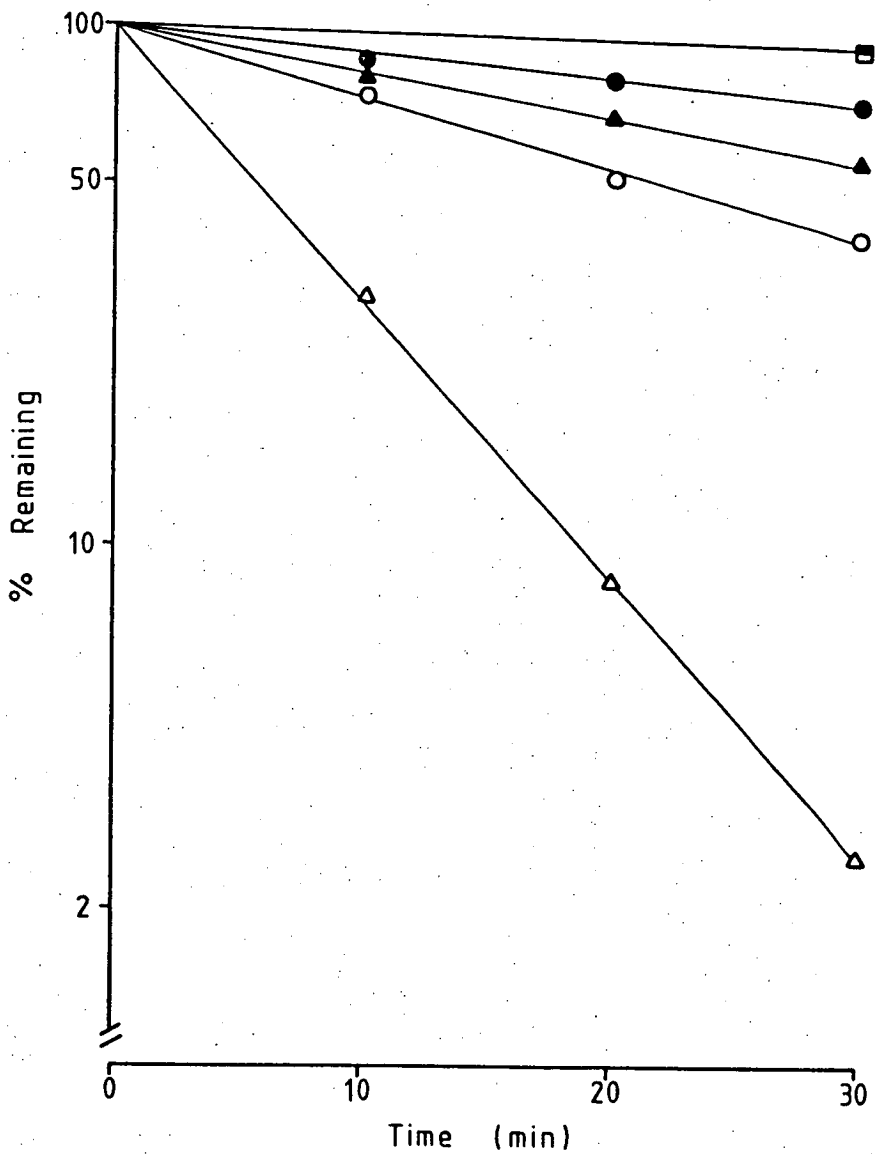


Figure 5.21 Metabolism of GTN (10 ng/ml) in suspensions of sheep liver (▲ △) and lung (● ○) tissue with (closed symbols) and without (open symbols) added GDNs (each at 100 ng/ml). Control incubations (■ □). Each point represents the mean of the results for two animals.

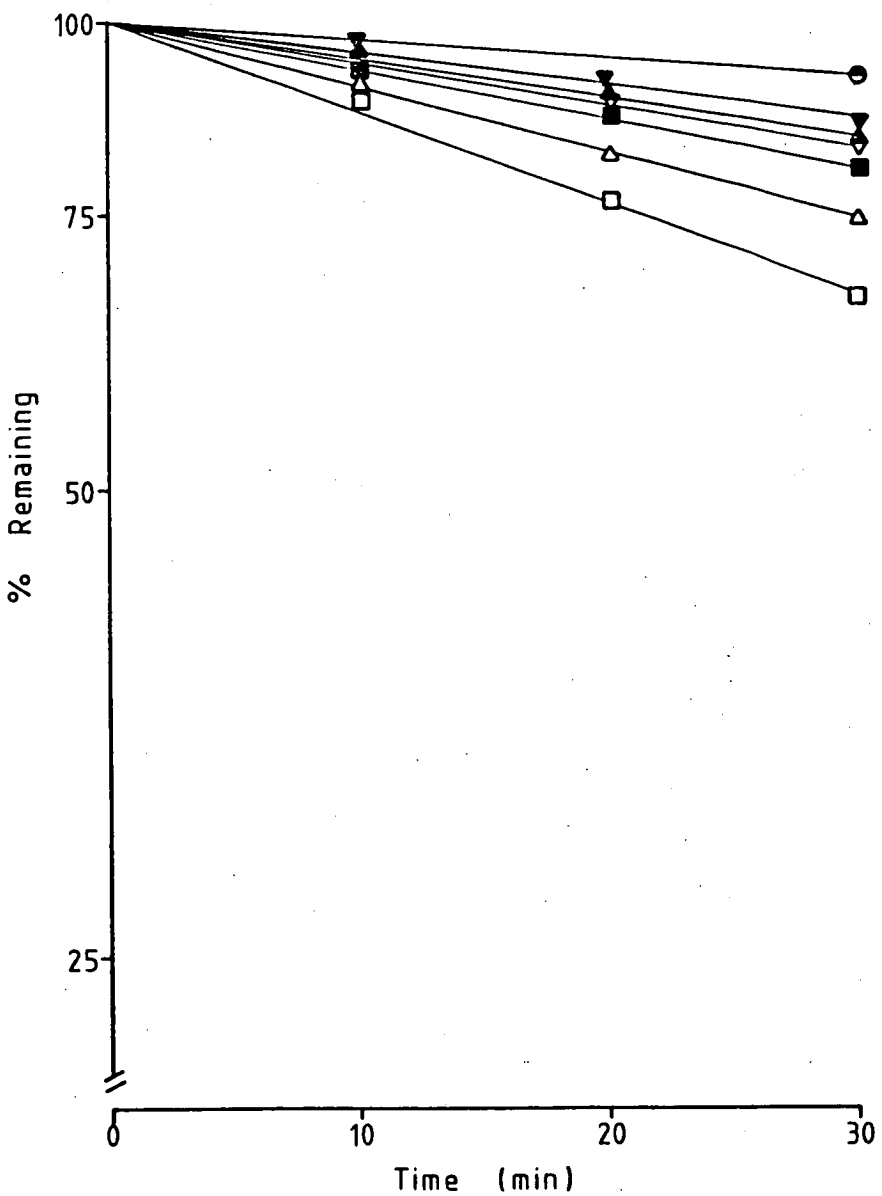


Figure 5.22 · Metabolism of GTN (10 ng/ml) in suspensions of sheep leg muscle (■ □), aorta (▲ △) and vena cava (▼ ▽) tissues with (closed symbols) and without (open symbols) added GDNs (each 100 ng/ml). Control incubations (● ○). Each point represents the mean of the reults for two animals.

Table 5.11 Percentage [a] of nitroglycerin (GTN) and its metabolites (GMNs, 1, 3-GDN and 1, 2-GDN) in sheep tissue homogenates after incubation for 30 minutes and the effect of added unlabelled GDNs (each 100 ng/ml) on the metabolism of GTN. (Data presented as the mean ± S.E., n = 3, for GTN 50 ng/ml but only the mean, n = 2, for GTN 10 ng/ml).

Initial Concentration (ng/ml)		Control		Liver		Lung		Muscle		Vena Cava		Aorta	
		-GDN	+GDN	-GDN	+GDN	-GDN	+GDN	-GDN	+GDN	-GDN	+GDN	-GDN	+GDN
GTN	50	95.3 (0.85)	96.1 (0.65)	2.7 (0.53)	58.2 (1.5)	41.5 (0.5)	72.4 (1.3)	69.1 (2.2)	80.8 (1.5)	73.8 (2.2)	87.7 (1.8)	85.3 (1.2)	88.7 (1.3)
1,3-GDN	0	2.3 (0.51)	2.2 (0.48)	37.2 (2.1)	16.3 (1.2)	17.7 (0.54)	9.7 (1.0)	16.3 (0.84)	10.0 (0.9)	16.3 (2.6)	8.0 (1.0)	7.3 (0.58)	5.9 (1.1)
1,2-GDN	0	2.3 (0.61)	1.7 (0.51)	52.7 (1.8)	25.4 (0.82)	38.0 (1.2)	17.3 (0.98)	13.7 (0.81)	9.1 (1.3)	10.1 (1.4)	4.1 (1.0)	7.1 (1.0)	5.4 (0.83)
GMNs	0	0 -	0 -	7.1 (0.81)	0.12 (0.08)	2.1 (0.04)	0.86 (0.04)	0.31 (0.09)	0 -	0 -	0 -	0 -	0 -
GTN	10	94.9	95.1	2.4	56.5	41.2	73.0	68.0	80.7	73.6	84.7	83.6	87.7
1,3-GDN	0	3.1	3.0	36.8	14.0	17.3	10.4	16.3	11.1	14.9	10.5	8.2	6.0
1,2-GDN	0	2.0	1.8	53.3	28.9	40.0	15.8	15.0	8.4	11.3	4.7	8.2	6.1
GMNs	0	0	0	6.9	0.30	1.9	0.75	0.48	0	0	0	0	0

[a] expressed as the percentage of the total radioactivity injected into the HPLC column.

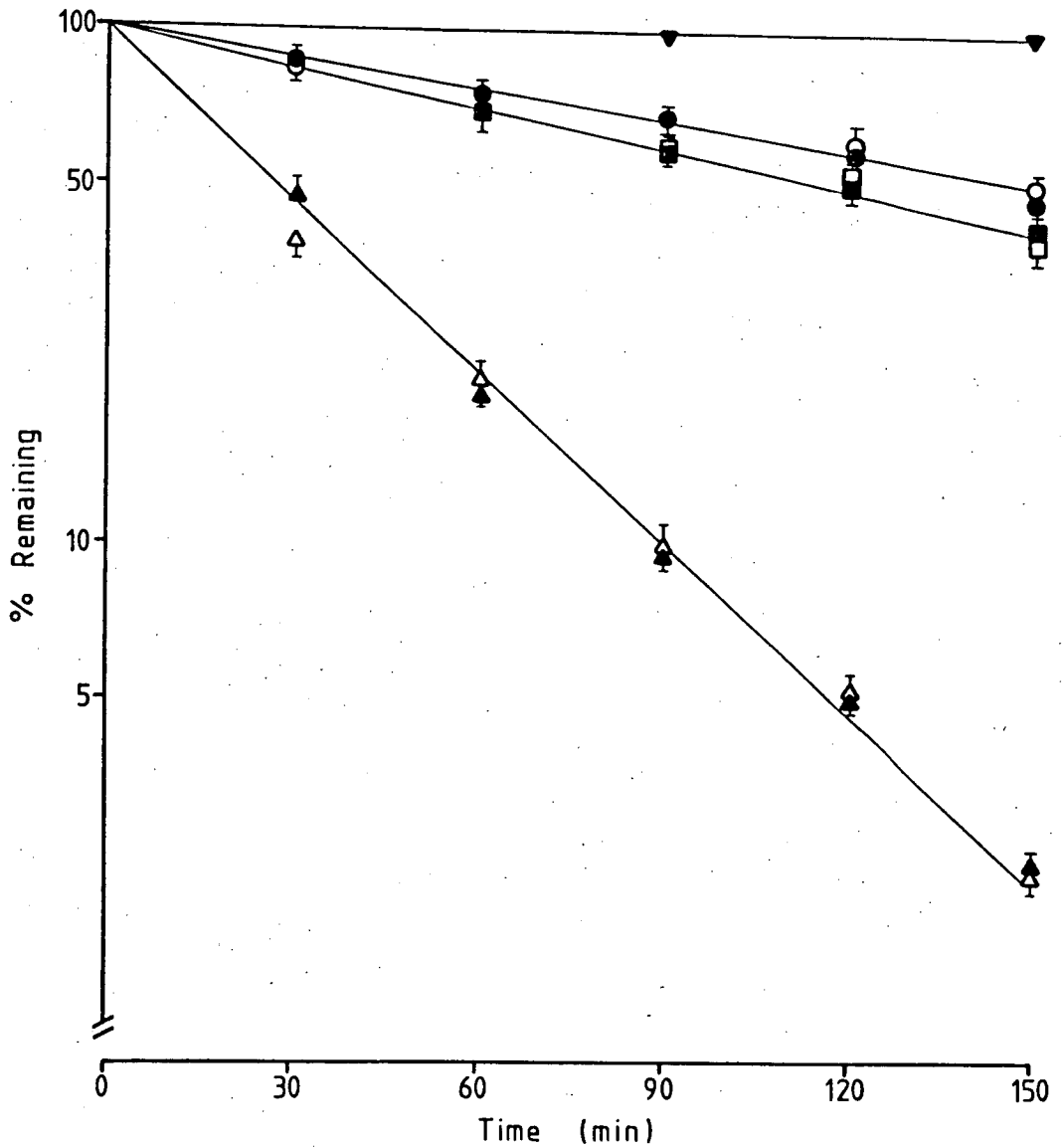


Figure 5.23 Metabolism of ASA (50 µg/ml) in suspensions of sheep liver (▲ △), leg muscle (■ □) and lung (● ○) tissue with (closed symbols) and without (open symbols) added SA (50 µg/ml). Control incubations (▼ ▽). Each point represents the mean ± se of the results for three animals.

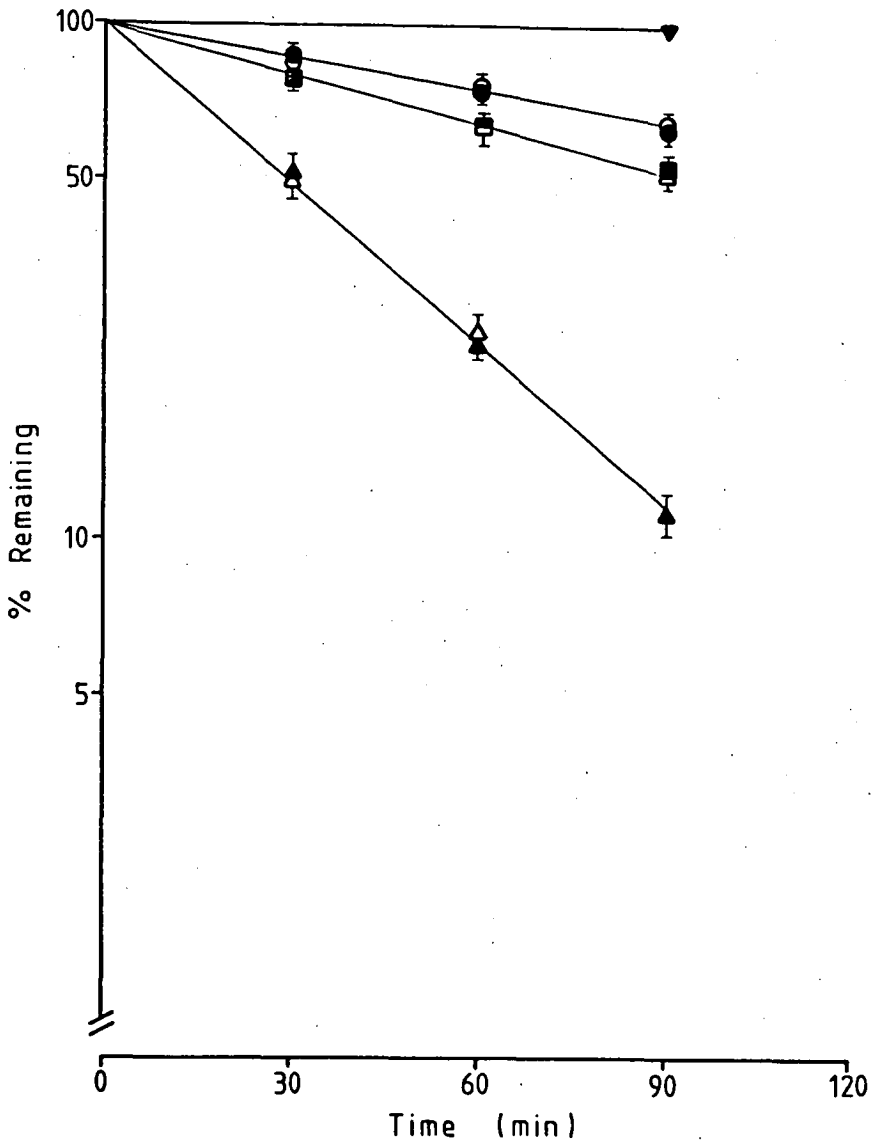


Figure 5.24 Metabolism of ASA (5 µg/ml) in suspensions of sheep liver (▲ △), leg muscle (■ □) and lung (● ○) tissue with (closed symbols) and without (open symbols) added SA (50 µg/ml). Control incubations (▼ ▽). Each point represents the mean ± se of the results for three animals.

Table 5.12 Percentage [a] of aspirin (ASA) and its metabolite (salicylic acid, SA) in sheep tissue homogenates after incubation for 90 min and the effect of added SA (50 µg/ml) on the metabolism of ASA (Mean (± S.E.), n = 3).

Initial ASA concentration (µg/ml)	Control		Liver		Lung		Muscle	
	-SA	+SA	-SA	+SA	-SA	+SA	-SA	+SA
50	99.7 (0.1)	99.7 (0.1)	10.3 (1.0)	9.6 (0.8)	68.3 (1.8)	64.4 (1.6)	59.3 (1.1)	58.0 (1.2)
-	0 -	-	88.9 (1.1)	-	31.3 (0.8)	-	40.1 (0.7)	-
5	99.8 (0.1)	99.7 (0.2)	11.3 (1.0)	11.2 (0.7)	64.3 (1.7)	63.8 (1.4)	57.2 (1.9)	59.1 (1.0)
-	0 -	-	86.0 (0.3)	-	36.0 (0.9)	-	42.9 (1.3)	-

[a] expressed as the percentage of ASA mass equivalents.

[b] only calculated for those samples not containing initial SA concentration of 50 µg/ml.

losses of GTN are observed in each of the homogenates studied - sheep liver, lung, leg muscle, vena cava and aorta. Metabolism of GTN to GDNs accounted for most of the loss (Table 5.11). Detectable amounts of GMNS were produced in liver, lung and muscle homogenates (Table 5.11). The rates of GTN loss from tissue homogenates appeared to be independent of the GTN initial concentrations employed (Figures 5.19 to 5.22).

The presence of 1,3-GDN and 1,2-GDN each at concentrations of about 100 ng/ml greatly reduced the rate of GTN metabolism by all tissue homogenates for both initial GTN concentrations studied (Figures 5.19 to 5.22, Table 5.11).

5.4. IN VITRO METABOLISM OF ASPIRIN IN SHEEP TISSUES

ASA was lost from homogenates of sheep liver, lung and leg muscle stored at 37°C with metabolism of ASA to SA quantitatively accounting (except for a small percentage in liver homogenates) for all ASA lost (Table 5.12). Figures 5.23 and 5.24 and Table 5.12 show that the rate of ASA loss in any given tissue appeared to be independent of the initial ASA concentrations employed.

The presence of SA added at a concentration of 50 µg/ml had negligible effect on the rate at which tissue homogenates metabolized ASA (Figures 5.23 and 5.24 and Table 5.12).

5.5. PHARMACOKINETICS AND PHARMACODYNAMICS OF BOLUS DOSES OF NITROGLYCERIN IN SHEEP

Simulated experiments showed that neither GTN nor its metabolites were sorbed by the plastic catheters used in these studies (Table 5.13).

Table 5.13 Recoveries of labelled glyceryl nitrates after passage through plastic catheters.

Drug	Drug Concentration (ng/ml)	Flow Rate (ml/min)	% Recovery (n = 1)
GTN	1.2	5	98.4
		40	99.7
1, 3-GDN	6.3	40	99.6
1, 2-GDN	7.8	40	100.3
GMNs	7.3	40	99.8

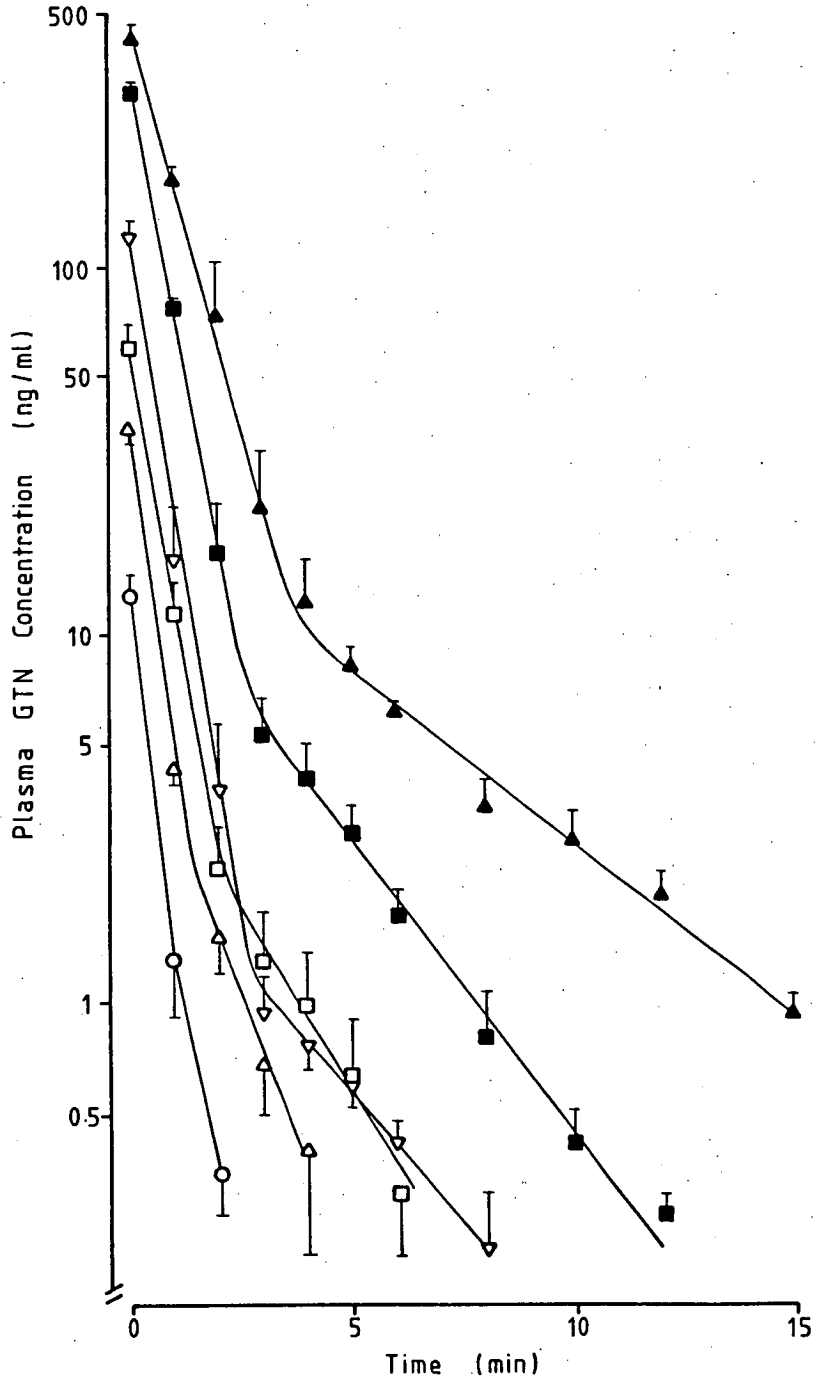


Figure 5.25 Plasma GTN concentrations in blood sampled from a femoral artery of sheep after bolus doses of GTN administered into the right femoral vein. (○) 2.7 μg/kg; (△) 5.4 μg/kg; (□) 13.6 μg/kg; (▽) 27.2 μg/kg; (■) 54.4 μg/kg; (▲) 108.8 μg/kg. Each point represents the mean ± se of the results for three sheep.

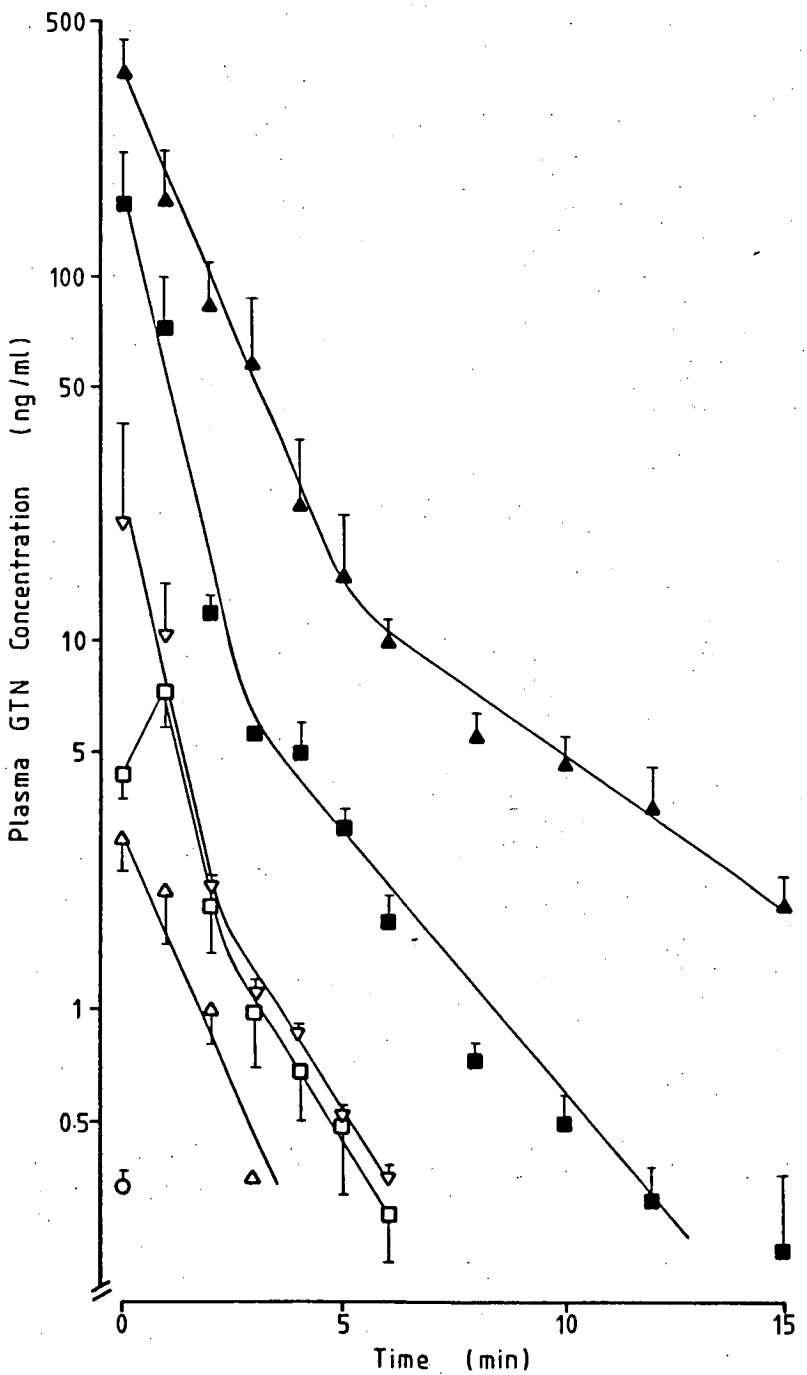


Figure 5.26 PLasma GTN concentrations in blood sampled from the right femoral vein of sheep after bolus doses of GTN administered into the left femoral vein. (○) 2.7 µg/kg; (△) 5.4 µg/kg; (□) 13.6 µg/kg; (▽) 27.2 µg/kg; (■) 54.4 µg/kg and (▲) 108.8 µg/kg. Each point represents the mean ± se of the results for three sheep.

Individual data for arterial and venous plasma GTN levels in three sheep that received bolus doses of GTN are presented in Tables A4 to A6. Mean data are presented in Figures 5.25 and 5.26. In both arterial and venous plasma, GTN concentrations decreased in a bi-exponential manner. The terminal half-lives showed an increase at the larger doses of GTN administered (Table 5.14). Figure 5.27 shows that peak GTN concentrations in arterial plasma increase proportionately with increasing dose of GTN; however, Figure 5.27 shows a disproportionate increase in peak venous plasma GTN concentrations with increasing doses of GTN. The dose dependence of plasma GTN concentrations is emphasized in Figure 5.28 which shows a relatively linear increase in the AUC for GTN in arterial plasma but a pronounced non-linear increase in the AUC for GTN in venous plasma. At the larger GTN doses the AUC for GTN in venous plasma exceeds that in arterial plasma (Figure 5.28) and this is emphasized in Table 5.14 where the venous availability ($F_{venous} = AUC_{venous}/AUC_{arterial}$) increases with increasing dose of GTN. As the dose of GTN is increased, the intrinsic clearance of GTN in the leg and the mean residence time of the drug in the leg decrease (Table 5.14).

Systemic clearance values for GTN in arterial and venous plasma are presented in Table 5.15 where it is evident that the arterial clearance, although variable, shows little overall reduction with increasing GTN dose. However, the venous systemic clearance shows a marked reduction for the two largest doses of GTN.

Individual data for peak % reductions in mean arterial blood pressure and mean left ventricular pressure in the same three sheep as above are presented as log GTN dose - response curves in Figures 5.29 and 5.30 (and Table A7) along with individual points to show that

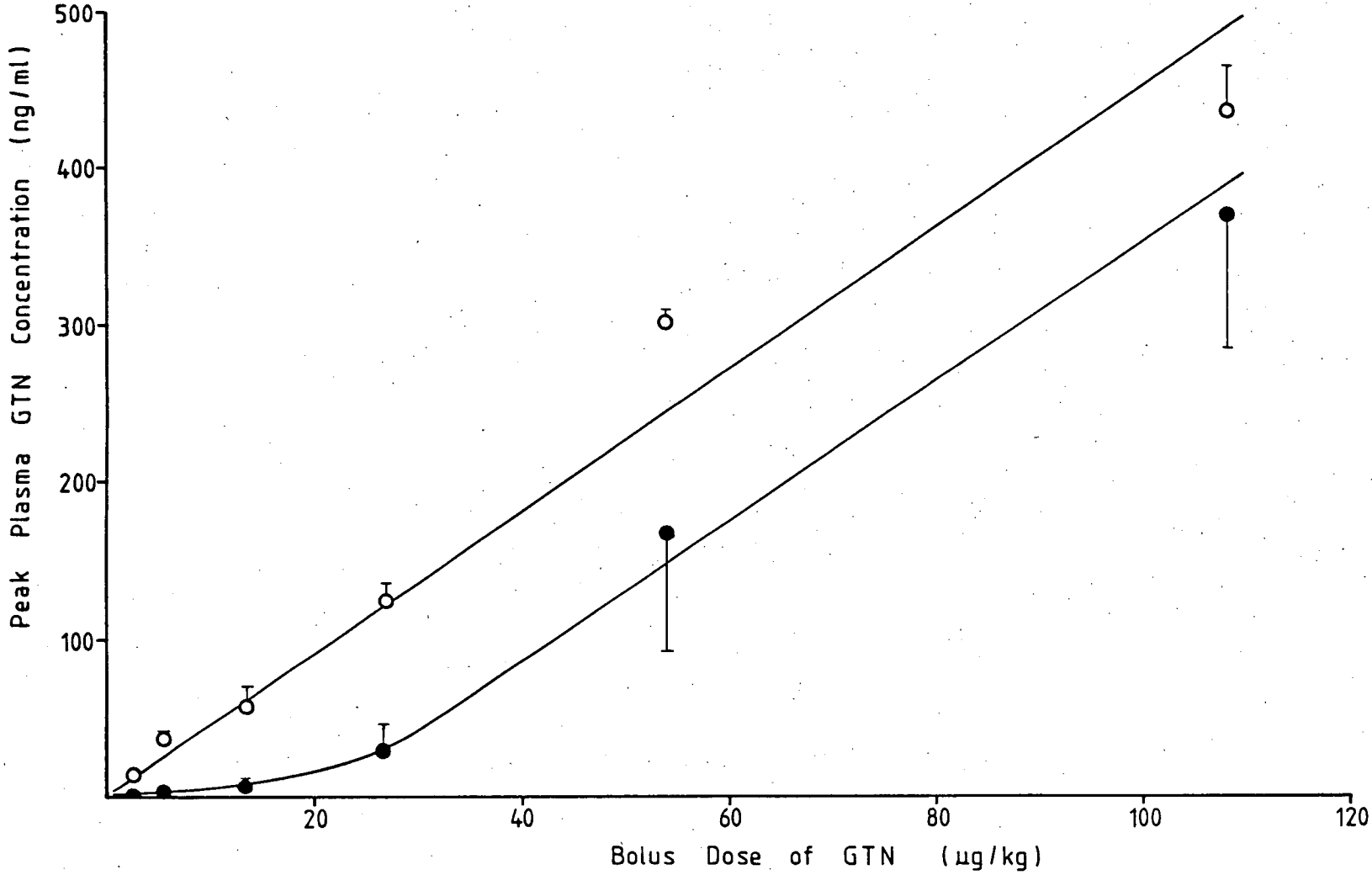


Figure 5.27 The relationship between bolus GTN doses and peak arterial (○) and venous (●) plasma GTN concentrations. Each point represents the mean ± se of the results for three sheep.

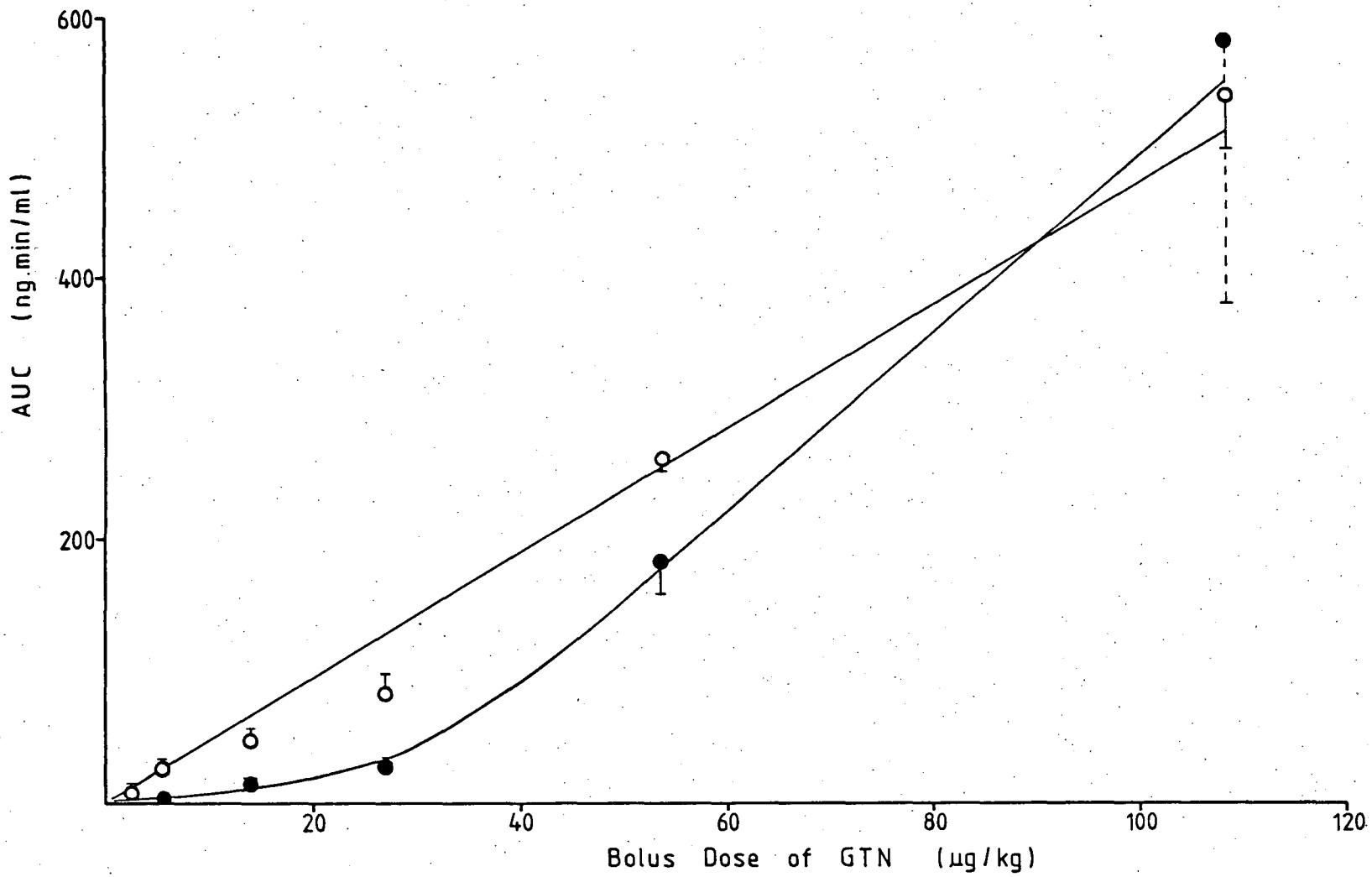


Figure 5.28 The relationship between bolus GTN doses and the area under the arterial (○) and venous (●) plasma GTN concentration-time profiles (AUC). Each point represents the mean ± se of the results for three sheep.

Table 5.14 Pharmacokinetic parameters of nitroglycerin (GTN) after bolus doses in sheep (Mean ± SE, n=3)

Dose (µg / kg)	Availability Across Leg	Intrinsic Clearance Across Leg (l/min)	Mean Residence Time (min)	Venous [a] Half-life (min)	Arterial [a] Half-life (min)
2.7	- [b]	-	-	-	1.13 ± 0.17
5.4	0.21 ± 0.03	2.70 ± 0.63	1.16 ± 0.19	1.13 ± 0.33	1.10 ± 0.35
13.6	0.29 ± 0.02	1.68 ± 0.23	1.10 ± 0.12	1.73 ± 0.32	1.80 ± 0.31
27.2	0.35 ± 0.12 [c]	1.61 ± 0.57	0.95 ± 0.29	2.13 ± 0.17 [c]	2.30 ± 0.35
54.4	0.70 ± 0.10 [c]	0.34 ± 0.18 [c]	0.46 ± 0.20 [c]	2.07 ± 0.26	2.13 ± 0.23 [c]
108.8	1.04 ± 0.27 [c]	0.16 ± 0.16 [c]	0.70 ± 0.16 [c]	3.20 ± 0.47 [c]	2.77 ± 0.35 [c]

[a] Calculated from the terminal phase of log GTN concentration-time profiles where detectability limits allowed.

[b] Insufficient data because of venous plasma GTN concentration detectability limits.

[c] Significantly different from the results obtained for the smallest dose administered.

Table 5.15 Systemic clearances (calculated as dose/AUC) of nitroglycerin after bolus doses in Sheep 13, 14 and 15. (Mean ± SE).

Dose (µg/kg)	Systemic Arterial Clearance (l/min)	Systemic Venous Clearance (l/min)
2.7	9.43 ± 1.18	-[a]
5.4	5.90 ± 0.61[b]	29.51 ± 5.30
13.6	8.50 ± 1.44	30.28 ± 6.83
27.2	9.40 ± 0.91	31.74 ± 6.40
54.4	5.93 ± 0.39[b]	8.71 ± 1.04[b]
108.8	5.86 ± 0.56[b]	6.42 ± 2.07[b]

[a]Plasma data did not allow calculation of AUC.

[b]Significantly different from the value for the smallest dose administered.

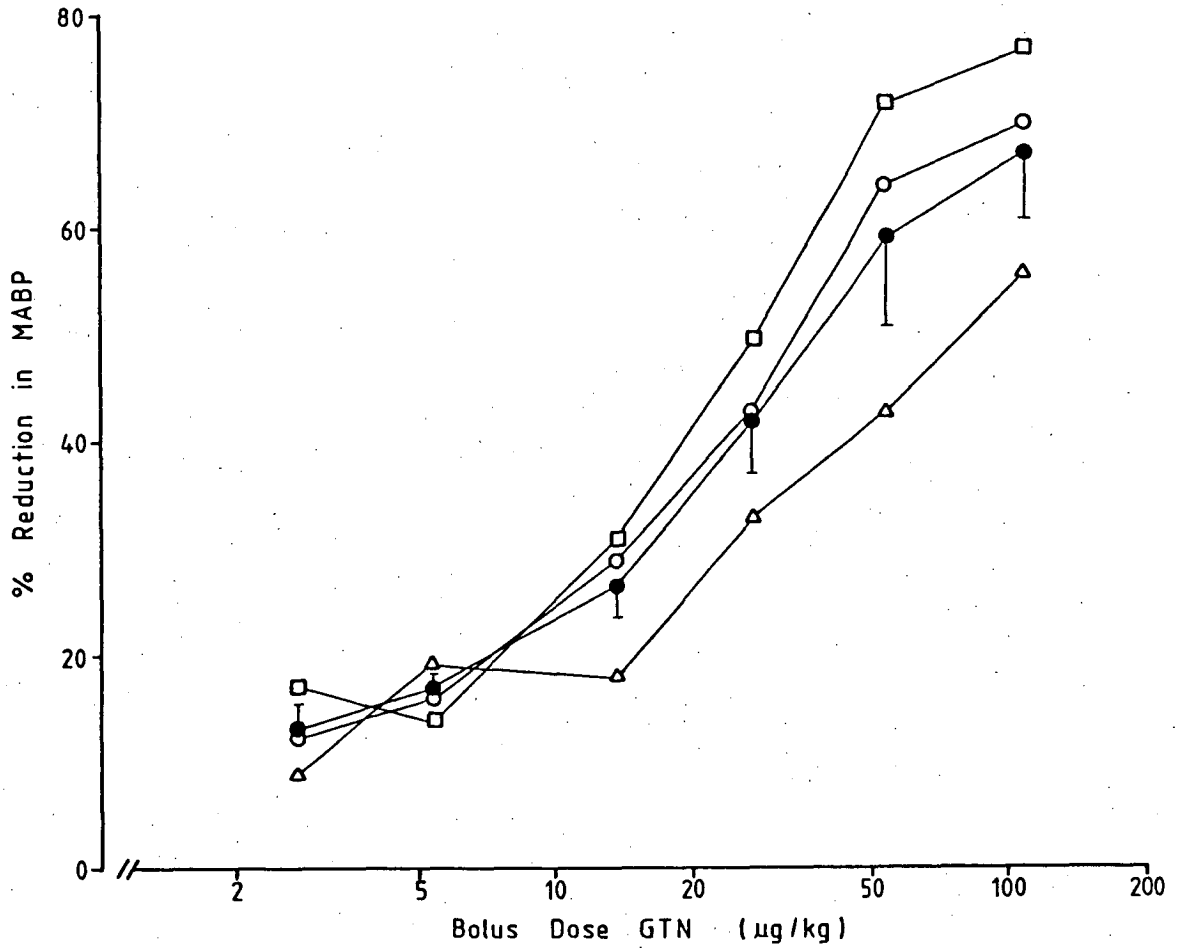


Figure 5.29 Log GTN dose-response curves for the effects of bolus doses of GTN on the mean arterial blood pressure (MABP) of Sheep 13 (○), 14 (△) and 15 (□). The mean ± se of the results for the three sheep is also plotted (●).

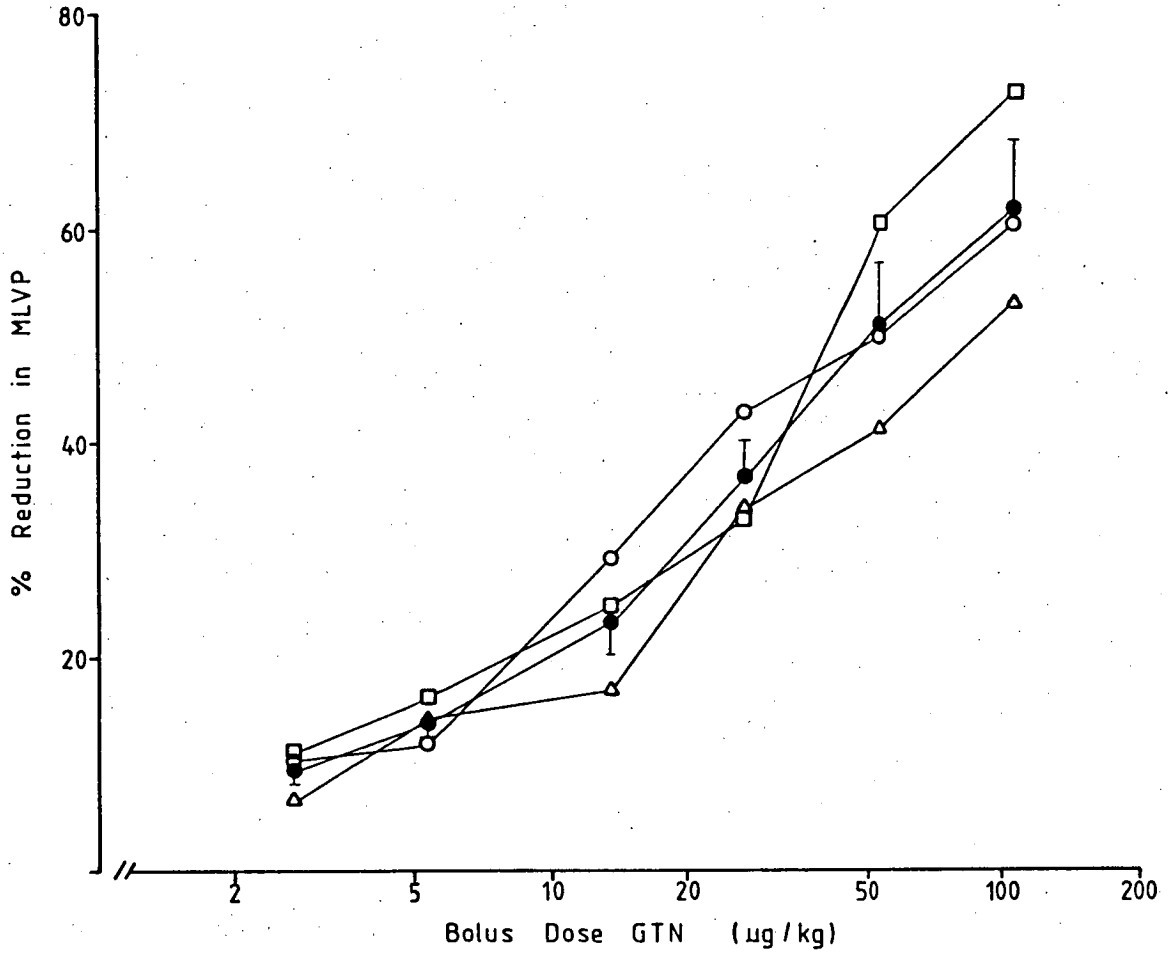


Figure 5.30 Log GTN dose-response curves for the effects of bolus doses of GTN on the mean left ventricular pressure (MLVP) of Sheep 13 (○), 14 (△) and 15 (□). The mean ± se of the results for the three sheep is also plotted (●).

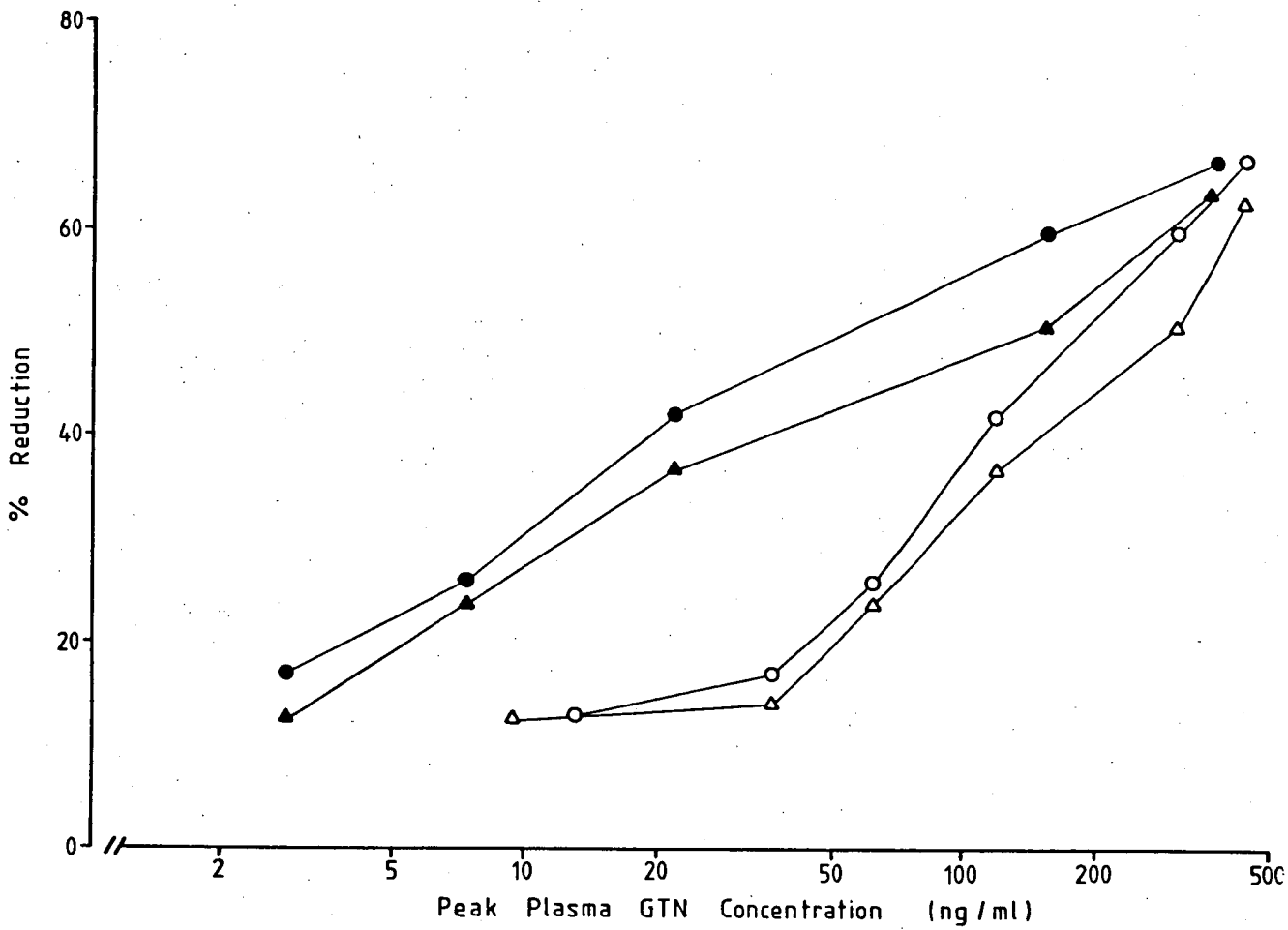


Figure 5.31 Relationship between the log of the peak arterial (open) and venous (closed symbol) plasma GTN concentration and the percent reduction in mean arterial blood pressure(○ ●) and mean left ventricular pressure (△ ▲) in sheep receiving bolus doses of GTN. Each point represents the mean of the results for 3 sheep.

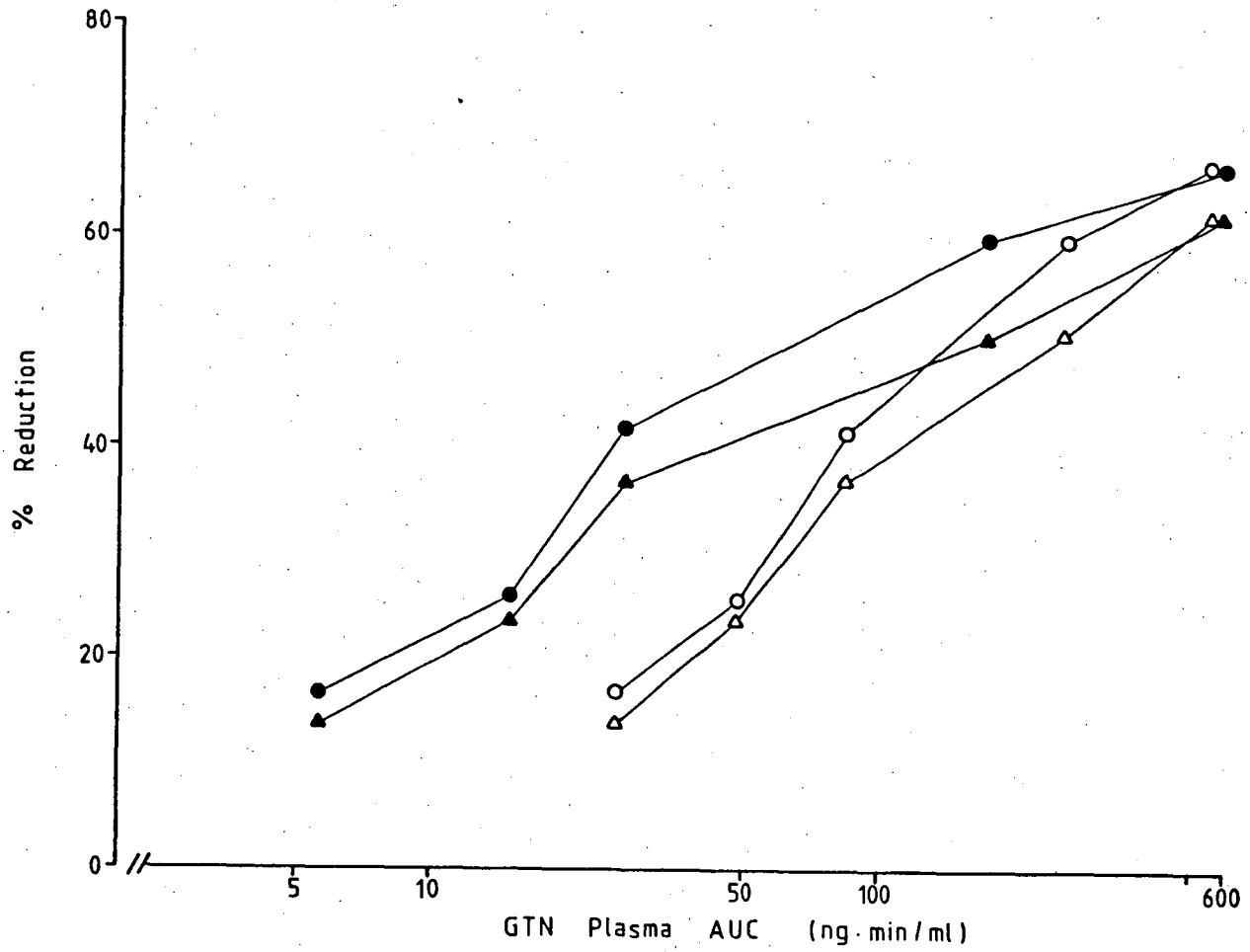


Figure 5.32 Relationship between the log of the area under the arterial (open symbols) and venous (closed symbols) plasma GTN concentration-time curves (AUC), and the percent reduction in mean arterial blood pressure (○ ●) and mean left ventricular pressure (△ ▲) in sheep receiving bolus doses of GTN. Each point represents the mean of the results for 3 sheep.

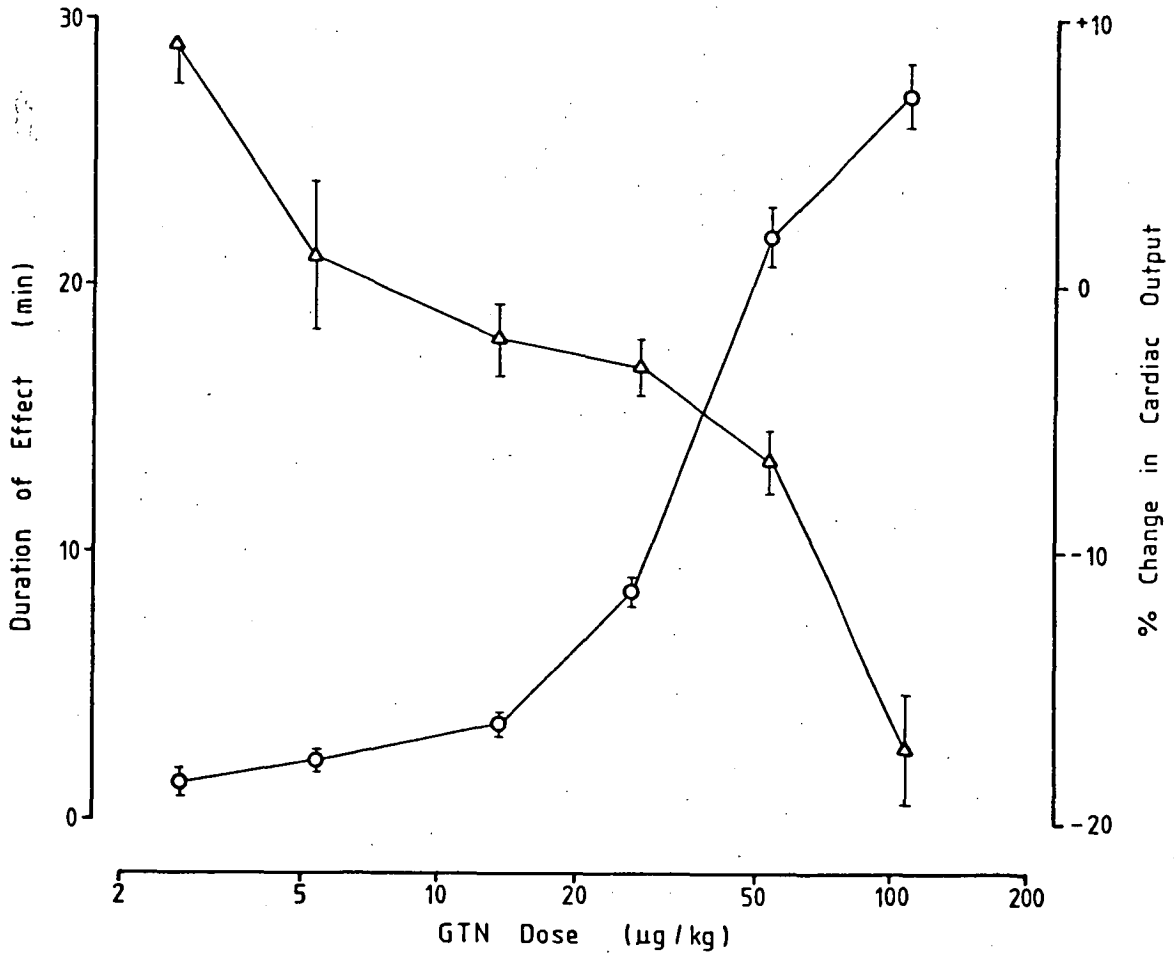


Figure 5.33 Log GTN dose-response curves for the effects of bolus doses of GTN on the duration of reduction in mean arterial blood pressure (○) and on cardiac output (△) in Sheep 13, 14 and 15. Each point represents the mean ± se of the results.

there were no discernible differences in responses to GTN for different dosing sequences. Two sheep (□ , △) received serially increasing doses of GTN while one sheep (o) received doses in a random fashion.

Relationships between % reduction in mean arterial blood pressure and left ventricular pressure versus the log of the peak arterial and peak venous plasma GTN concentrations are seen in Figure 5.31. Similar relationships are seen in Figure 5.32 for GTN plasma AUCs.

Figure 5.33 (and Table A8) shows that increasing bolus doses of GTN result in a greater duration of reduction in mean arterial blood pressure (i.e., time for blood pressure to return from maximal reduction to baseline). When these results are studied along with the results presented in Figure 5.29, it is evident that increasing bolus doses of GTN result in greater reductions in mean arterial blood pressure which have a longer duration of depression. Both of these effects are also associated with changes in cardiac output (Figure 5.33). Small bolus doses of GTN caused a slight increase in cardiac output but the larger doses resulted in the cardiac output falling. The duration of the fall in cardiac output was always less than the duration of the fall in blood pressure.

5.6. PHARMACOKINETICS AND PHARMACODYNAMICS OF NITROGLYCERIN AFTER CONTINUOUS INFUSIONS IN SHEEP

5.6.1. Nitroglycerin Kinetics and Dose-Response Relationships

Tables A9 to A17 and Figures 5.34, 5.35 and 5.36 present individual and mean data respectively for plasma GTN concentrations in blood samples taken from different sites in the sheep receiving con-

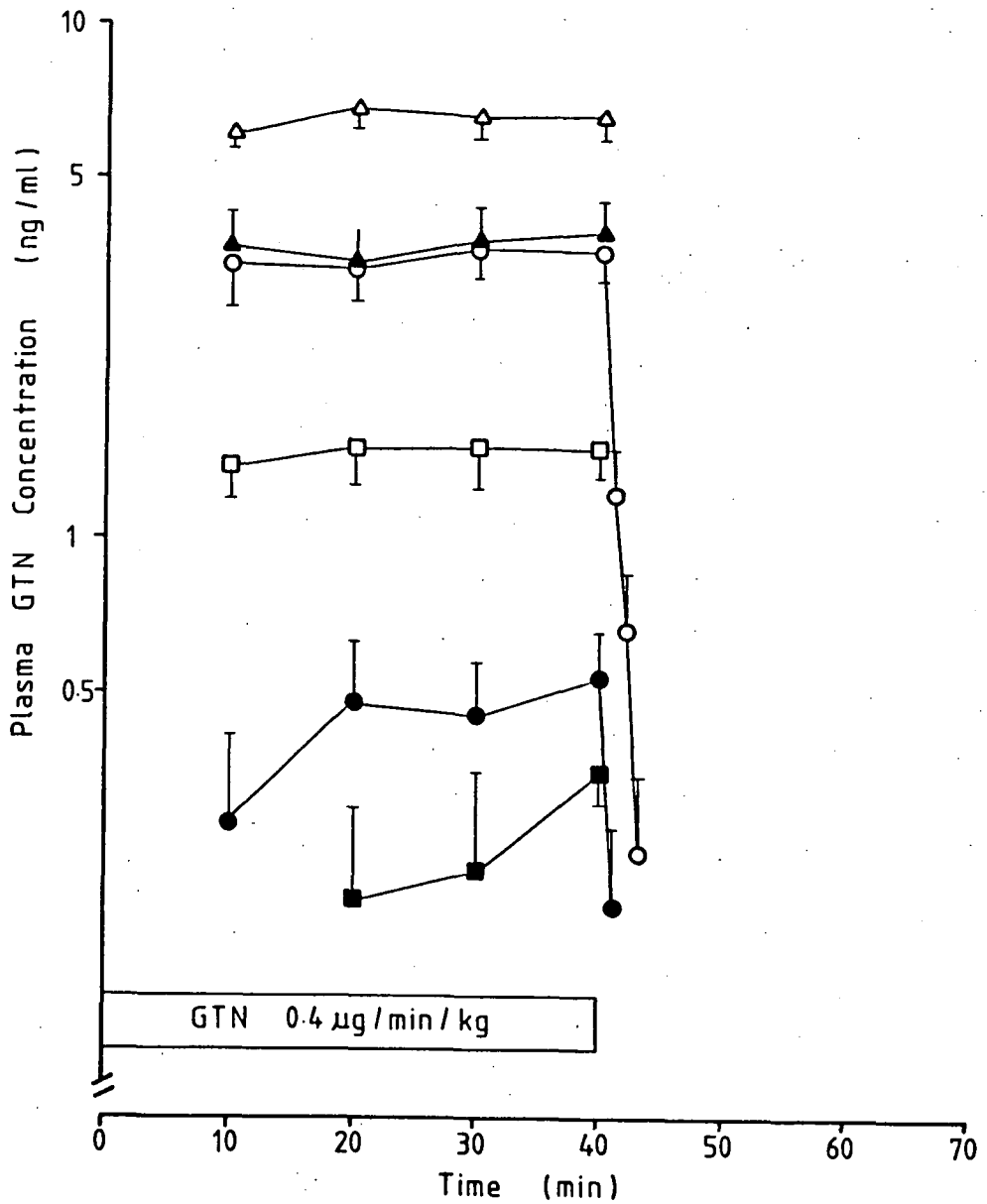


Figure 5.34 Plasma GTN concentration-time profiles after continuous infusions of GTN into the right femoral vein of Sheep 9, 16 and 17. (△) pulmonary artery; (▲) left ventricle; (○) femoral artery; (●) left femoral vein; (□) portal vein; (■) hepatic vein. Each point represents the mean ± se.

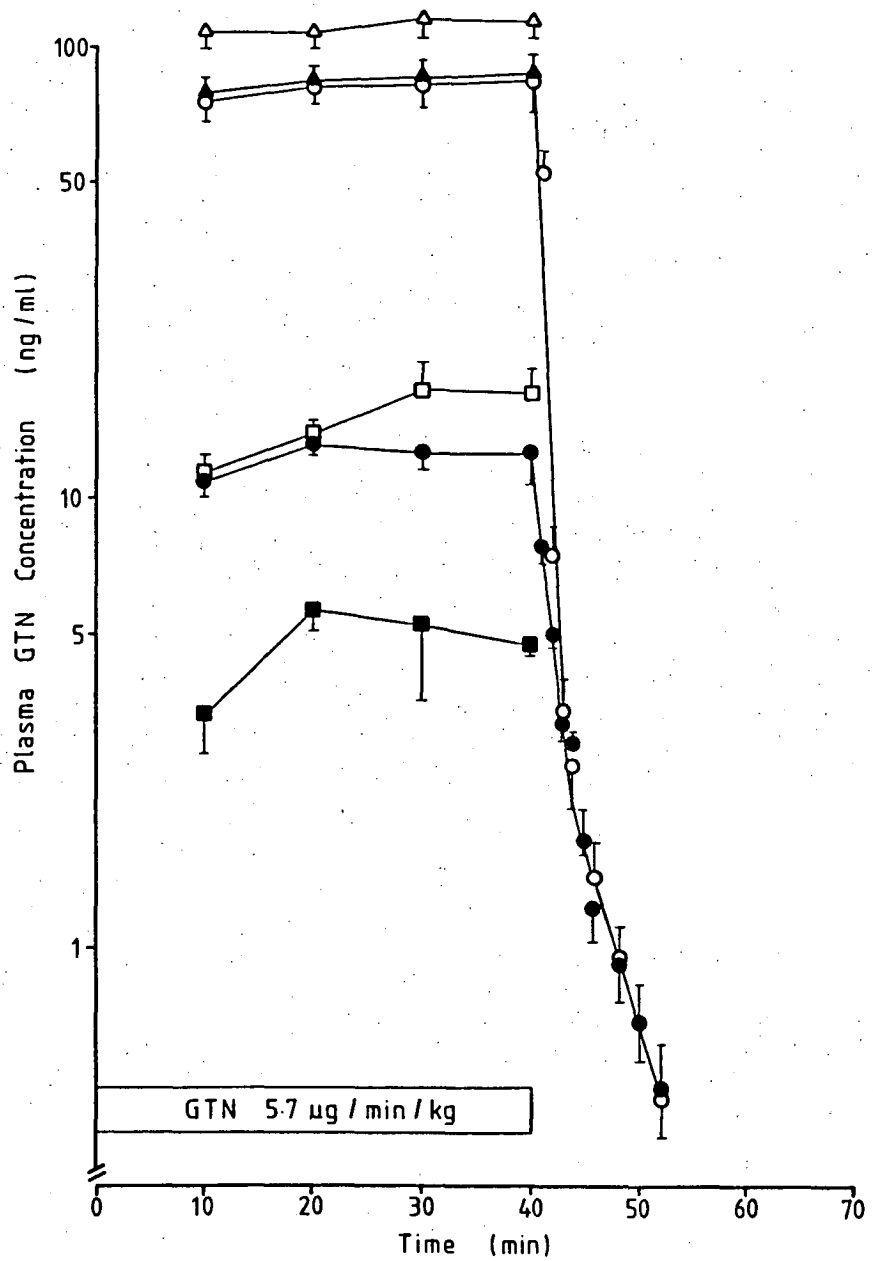


Figure 5.35 PLasma GTN concentration-time profiles after continuous infusions of GTN into the right femoral vein of Sheep 10, 11 and 12. (△) pulmonary artery; (▲) left ventricle; (○) femoral artery; (●) left femoral vein; (□) portal vein; (■) hepatic vein. Each point represents the mean ± se.

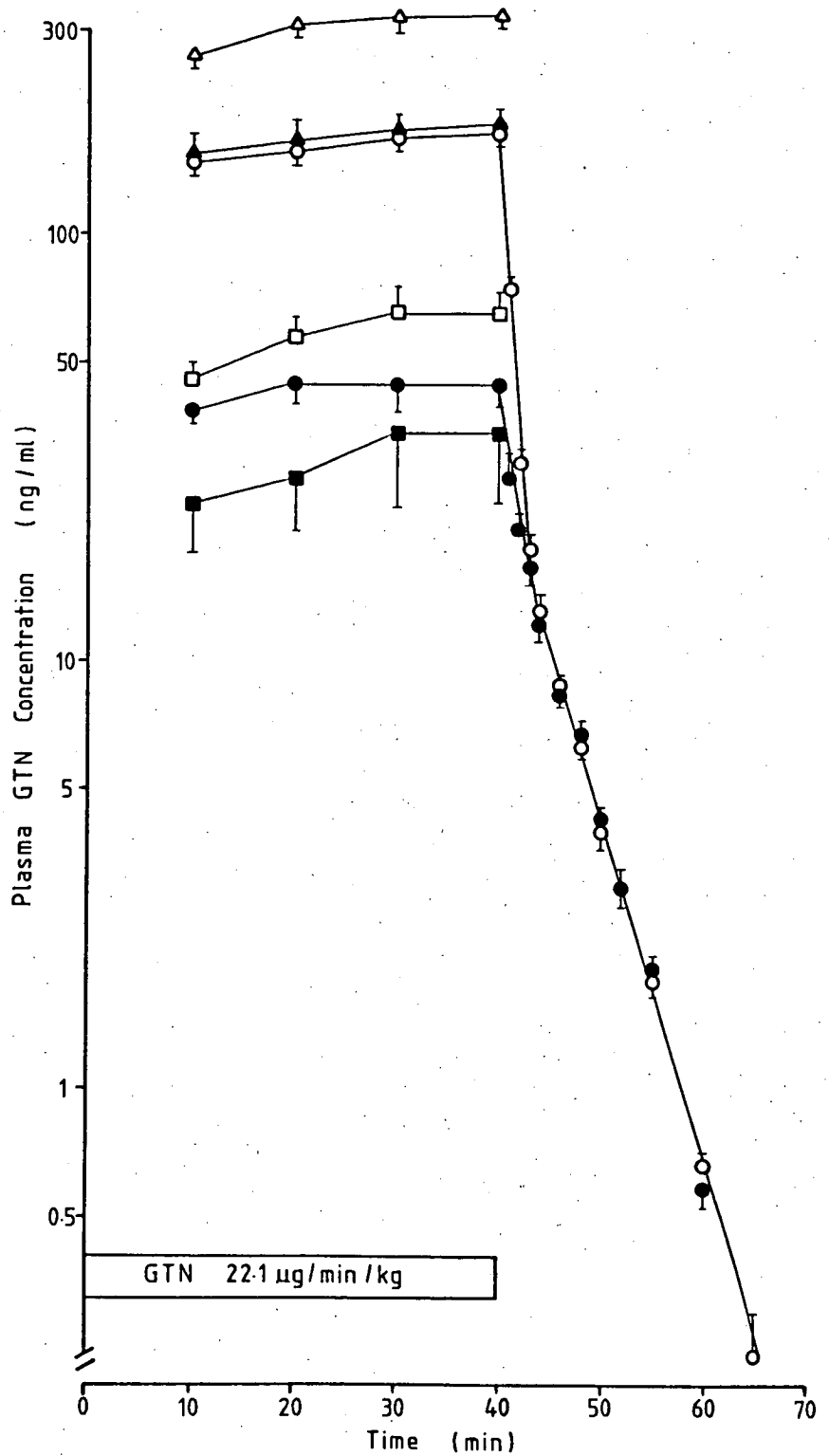


Figure 5.36 Plasma GTN concentration-time profiles after continuous infusions of GTN into the right femoral vein of Sheep 18, 19 and 20. (△) pulmonary artery; (▲) left ventricle; (○) femoral artery; (●) left femoral vein; (□) portal vein; (■) hepatic vein. Each point represents the mean ± se.

Table 5.16 Pharmacokinetic parameters of nitroglycerin (GTN) after continuous intravenous infusions in sheep (Mean ± SE, n=3).

GTN Infusion Rate (µg/min/kg)	Availability			Intrinsic Clearance (l/min)			Mean Residence Time (min)			Terminal Half-life[a] (min)
	leg	lung	liver	leg	lung	liver	leg	lung	liver	
0.4	0.12 ± 0.02	0.58 ± 0.05	0.13 ± 0.03	4.07 ± 0.53	4.14 ± 0.76	10.6 ± 3.6	3.57 ± 1.18	0.16 ± 0.12	10.0 ± 3.9	-[c]
5.7	0.17 ± 0.02	0.72[b] ± 0.02	0.31[b] ± 0.03	3.18 ± 0.33	2.40[b] ± 0.13	3.70[b] ± 0.88	1.68 ± 1.00	0.07 ± 0.08	0 0	3.02 ± 0.19
22.1	0.29[b] ± 0.04	0.53 ± 0.04	0.49[b] ± 0.09	1.64[b] ± 0.17	5.41 ± 1.06	1.90[b] ± 0.48	0.95 ± 0.09	0 0	0.24[b] ± 0.08	3.58 ± 0.33

[a] Calculated from the terminal phase of log GTN concentration-time profiles where detectability limits allowed.
[b] Significantly different from the values for the smallest dose administered.
[c] Insufficient data because of venous plasma GTN concentration detectability limits.

tinuous IV infusions of GTN at rates of 0.4, 5.7 and 22.1 μg/min/kg. For each infusion rate studied, apparent steady-state plasma GTN concentrations were generally achieved within 20 min of the commencement of GTN infusion (Figures 5.34 to 5.36).

There was an approximate linear relationship between the GTN infusion rate and the apparent steady-state plasma GTN concentration in blood samples taken from all sampling sites (Figure 5.37). This relationship is translated into an approximate linear increase in plasma AUCs (Figure 5.38).

On termination of an infusion, femoral venous and arterial plasma concentrations fell rapidly in a bi-exponential manner. The two phases are evident in Figures 5.35 and 5.36 but not in Figure 5.34 where the plasma GTN concentrations fell rapidly below detectable limits (0.2 ng/ml). The terminal half-life, when measurable, was found to be 3-4 min (Table 5.16).

The GTN concentration in the plasma of blood leaving an organ (i.e., hepatic vein, left ventricle, femoral vein) was substantially less than that in the plasma of blood entering the organ (i.e., portal vein, pulmonary artery, femoral artery) (Figures 5.34 to 5.36). Losses of GTN during passage through each organ were in the rank order hind-leg>liver>lung. This is also evident from Table 5.16 which shows that the availability (calculated as the ratio of AUCS of plasma GTN concentrations leaving an organ to those entering the organ) is in the same rank order.

Table 5.16 also shows that the intrinsic clearance for a given vascular bed varies from organ to organ for each infusion rate. At greater GTN infusion rates, the availability of GTN across the leg and liver appears to increase. A concomitant decrease in the clearance of

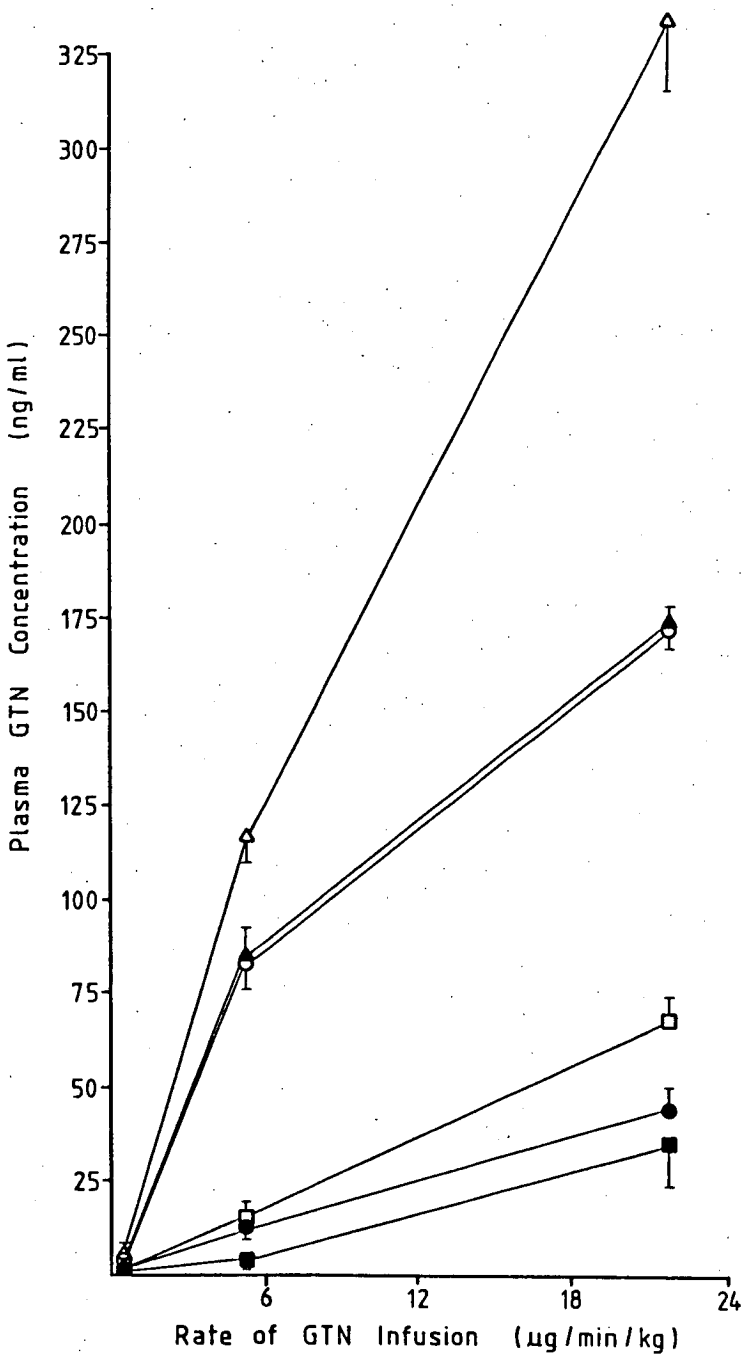


Figure 5.37 Apparent steady-state plasma GTN concentrations resulting from continuous intravenous infusions of GTN in sheep. (△) pulmonary artery; (▲) left ventricle; (○) femoral artery; (●) femoral vein; (□) portal vein; (■) hepatic vein. Each point represents the mean ± se of the results for 3 sheep.

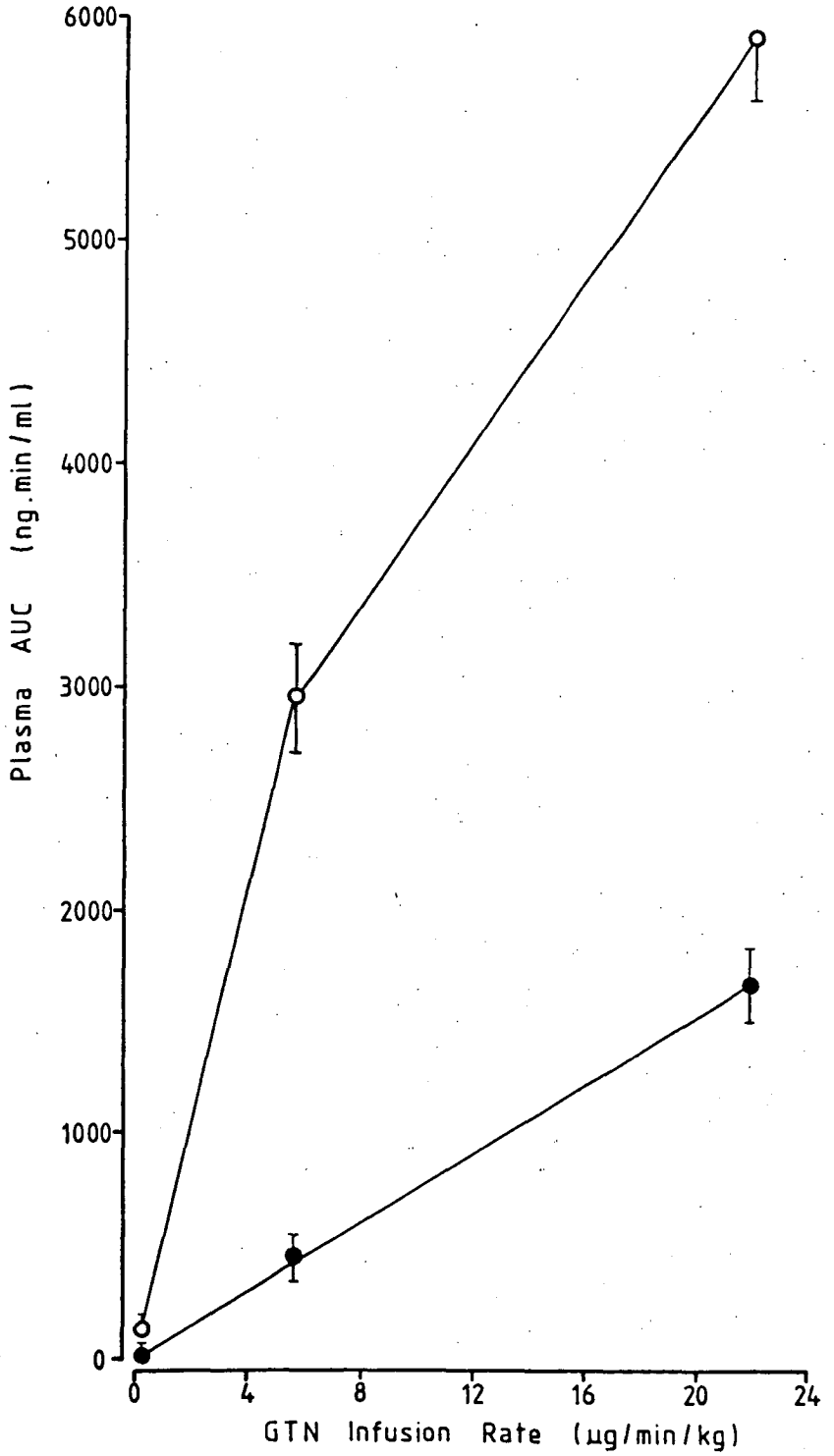


Figure 5.38 Plasma AUCs for GTN resulting from continuous intravenous infusions of GTN in sheep. (○) femoral artery; (●) femoral vein. Each point represents the mean ± se of the results for 3 sheep.

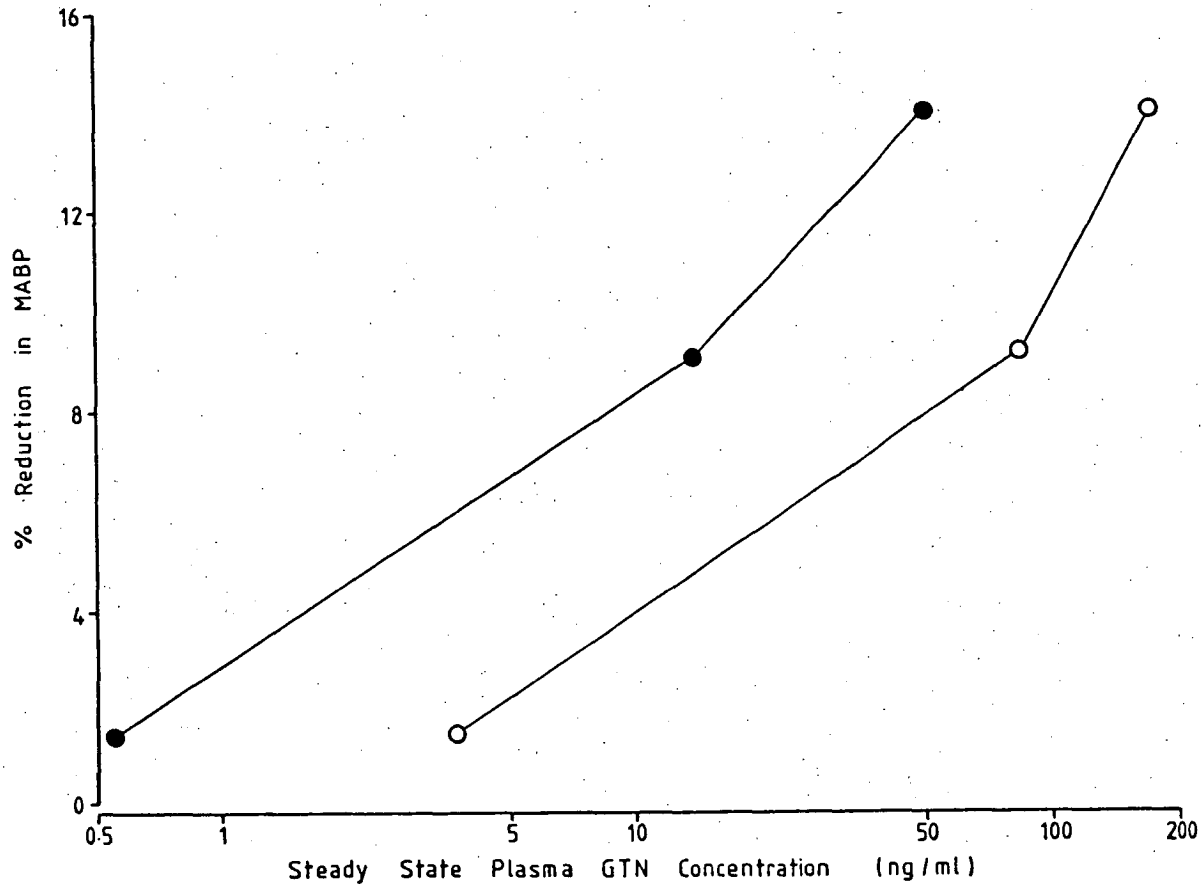


Figure 5.39 Relationship between the log of the apparent steady state arterial (○) and venous (●) plasma GTN concentrations and the percent reduction in mean arterial blood pressure (MABP) in sheep receiving continuous intravenous infusions of GTN. Each point represents the mean of the results for 3 sheep.

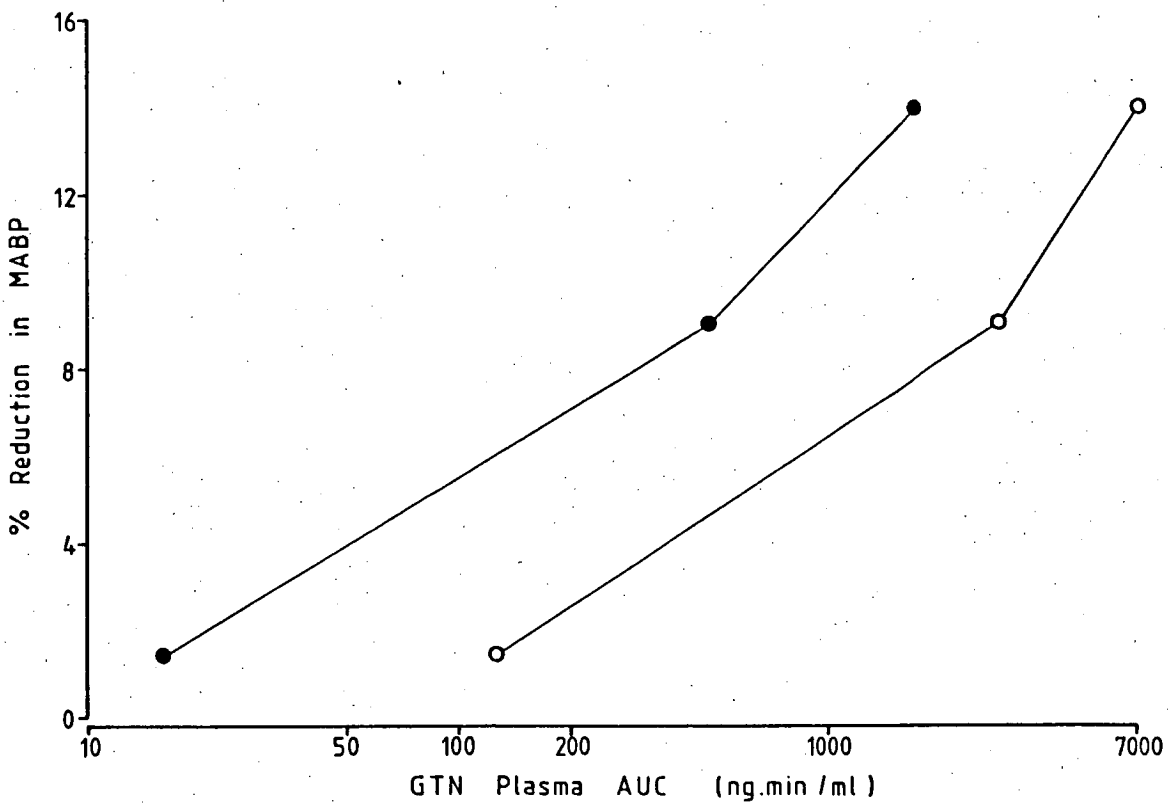


Figure 5.40 Relationship between the log of the area under the arterial (○) and venous (●) plasma GTN concentration-time profiles (AUC) and the percent reduction in mean arterial blood pressure (MABP) in sheep receiving continuous intravenous infusions of GTN. Each point represents the mean of the results for 3 sheep.

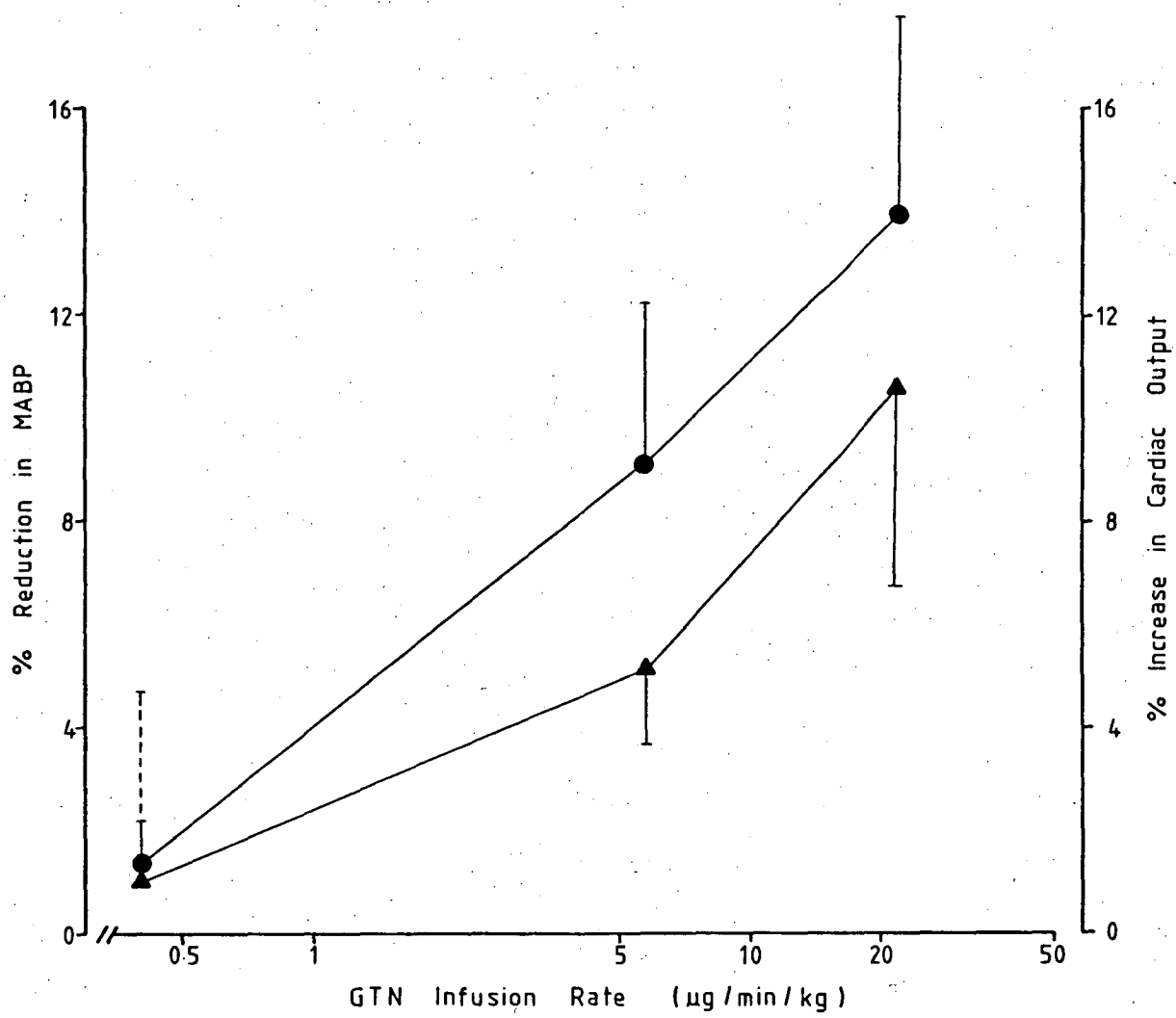


Figure 5.41 Log GTN dose-response curves for the effects of continuous intravenous infusions of GTN on mean arterial blood pressure (MABP) (●), and on cardiac output (▲). Each point represents the mean ± se of the results for 3 sheep.

GTN across the leg and liver is observed at the higher doses whereas the availability and clearance of GTN in the lung is increased and decreased, respectively, at the middle GTN dose (Table 5.16). Mean residence times of GTN in leg, lung and liver all decrease with increasing doses of GTN (Table 5.16). Systemic clearances calculated using arterial plasma GTN concentrations were much less than those calculated for venous plasma data (Table 5.18).

Figures 5.39 and 5.40 show the relationship between log peak GTN plasma concentrations and % reduction in MABP, and log GTN plasma AUC and % reduction in MABP for continuous GTN infusions. Figure 5.41 and Table A52 show that increasing the dose (or rate of administration) of GTN results in a greater reduction in MABP and a greater increase in cardiac output.

5.6.2 Effect of Glyceryl Dinitrates on Nitroglycerin Pharmacokinetics

Bolus doses of GDNs (5 mg total of 1,3-GDN and 1,2-GDN) had a marked effect on the pharmacokinetics of GTN in two sheep receiving continuous IV infusions of GTN at a rate of 5.7 μg/min/kg (Figures 5.42 and 5.43 and Tables A18 and A19). It can be seen that the venous plasma GTN concentrations are greater than the arterial plasma GTN concentrations after administration of 5 mg GDNs in the post-infusion stage. In sheep receiving the same dose of GTN without a bolus dose of GDNs being administered (Figure 5.35) the profile of venous plasma GTN concentrations is always less than or equal to the arterial profile after termination of the GTN infusion. Figures 5.42 and 5.43 also show that the terminal phases of GTN kinetics is extended after GDN dosing (Table 5.17) (6-10 min after GDNs compared to about 3 min without GDNs).

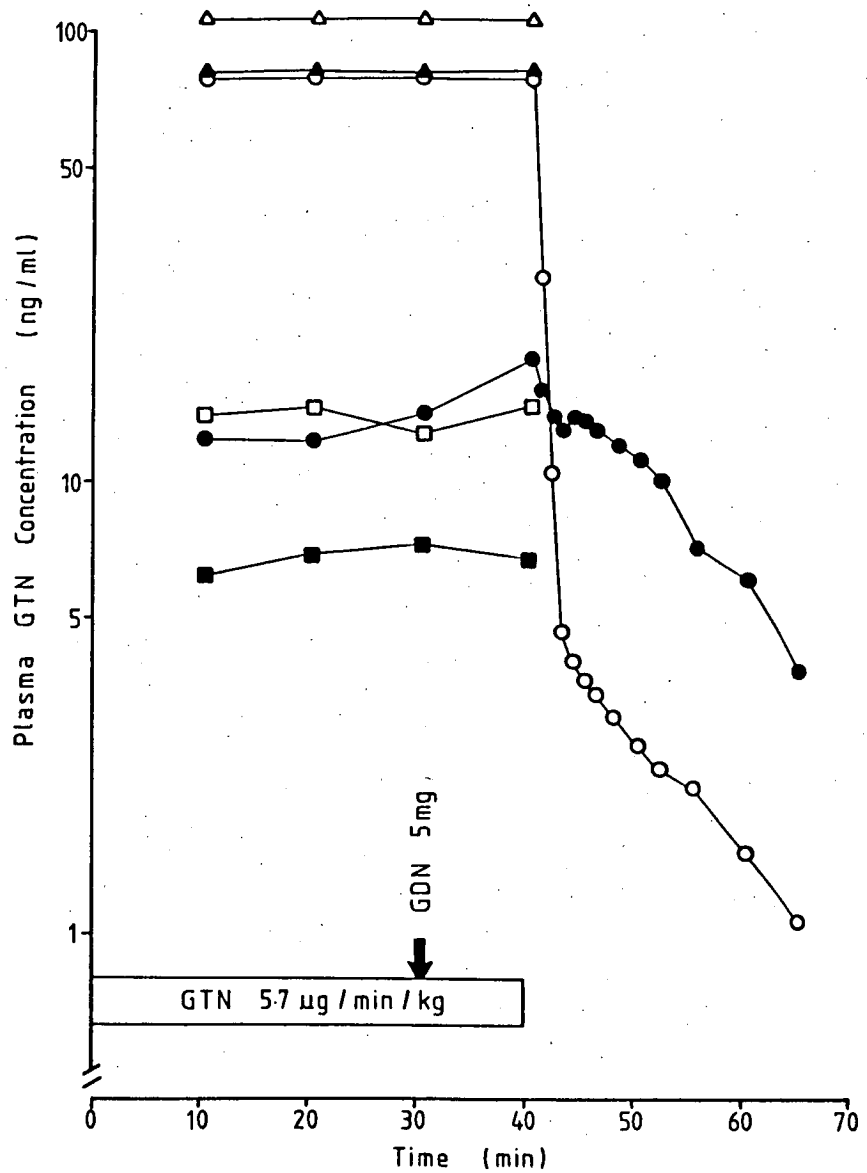


Figure 5.42 The effects of a bolus dose of GDNs (5 mg total of 1,3-GDN and 1,2-GDN) on the pharmacokinetics of GTN in Sheep 29 receiving a continuous intravenous infusion of GTN. (△) pulmonary artery; (▲) left ventricle; (○) femoral artery; (●) left femoral vein; (□) portal vein; (■) hepatic vein.

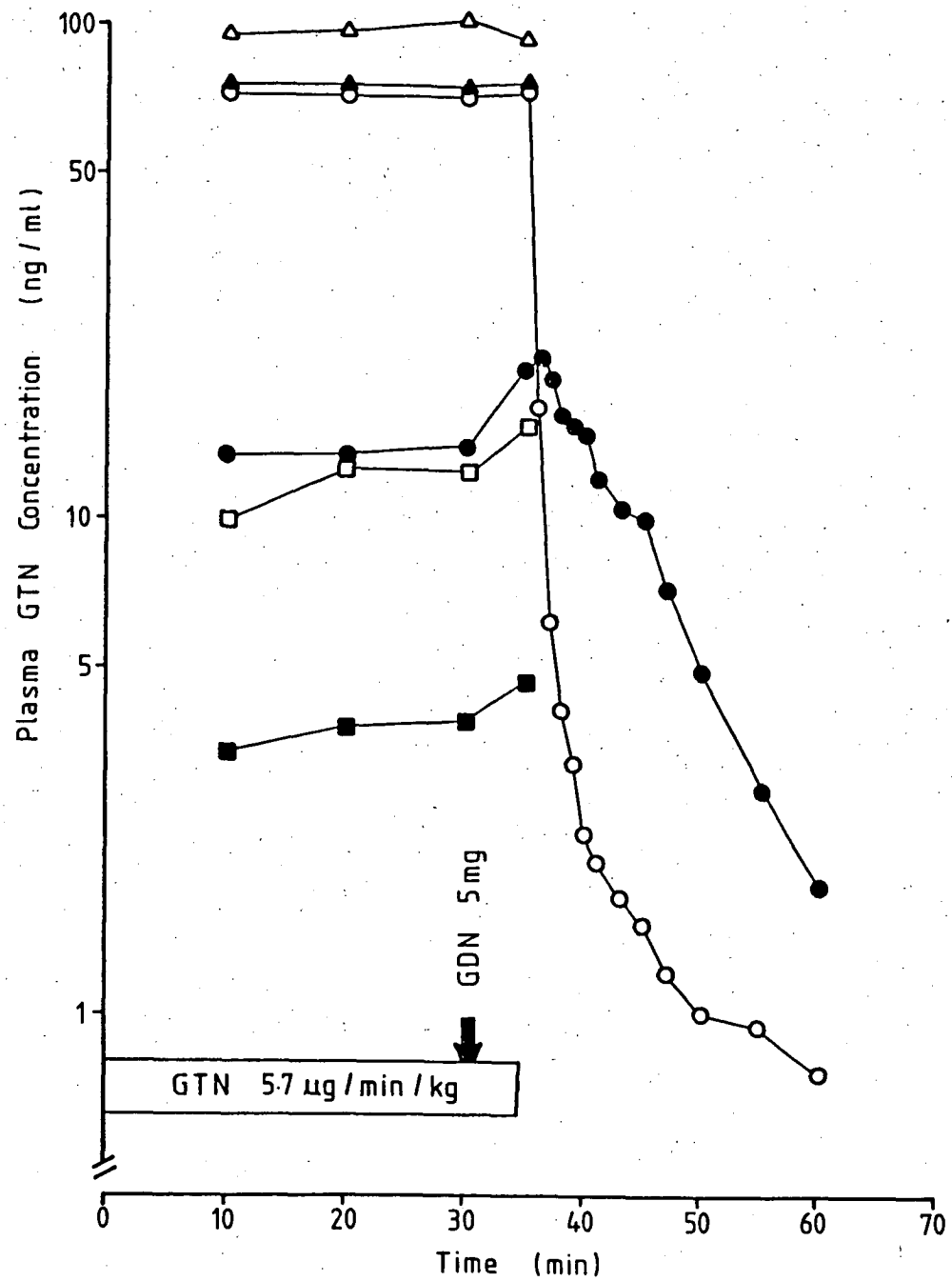


Figure 5.43 The effects of a bolus dose of GDNs (5 mg total of 1,3-GDN and 1,2-GDN) on the pharmacokinetics of GTN in Sheep 30 receiving a continuous intravenous infusion of GTN. (△) pulmonary artery; (▲) left ventricle; (○) femoral artery; (●) left femoral vein; (□) portal vein; (■) hepatic vein.

Table 5.17 Effect of glyceryl dinitrates (GDN) on the pharmacokinetics of nitroglycerin (GTN) after continuous intravenous infusions of GTN in sheep.

GTN Infusion Rate (μg/min/kg)	Availability	Intrinsic Clearance (l/min)	Mean Residence Time (min)	Terminal[a] Half-life (min)
5.7	0.20	2.55	3.65	2.65
	0.14	3.31	0.47	3.10
	0.16	3.67	0.93	3.30
5.7 + 5 mg GDN	0.26	1.61	11.90	10.00
	0.28	1.54	7.21	6.25

[a]Calculated from the terminal phase of log GTN venous plasma concentration-time profiles.

Table 5.18 Individual systemic clearances (calculated as dose/AUC) of nitroglycerin (GTN) after continuous intravenous infusions in sheep.

GTN Infusion rate (µg/min/kg)	Systemic arterial clearance (l/min)	Systemic venous clearance (l/min)
0.4	3.2	21.0
	7.0	71.8
	4.7	32.6
5.7	2.7	13.3
	2.3	16.2
	2.9	20.7
5.7 + 5 mg GDN Bolus	2.1	10.5
	2.5	8.8
22.1	5.4	21.2
	5.5	15.0
	4.0	16.3

Table 5.17 shows that the availability of GTN in the leg increases after the administration of GDNs (average availability without GDNs is 0.17, average availability after GDNs is 0.27) and the intrinsic clearance of GTN in the leg is concomitantly reduced (average without GDNs is 3.2 1/min, average after GDNs is 1.6 1/min). The mean residence time of GTN in the leg is also markedly changed after GDN dosing (average 1.7 min without GDNs, average 9.5 min after GDNs).

The average systemic arterial clearance of GTN is only marginally reduced after GDNs (2.6 1/min without GDNs, 2.3 1/min after GDNs) however, the average systemic venous clearance of GTN was greatly reduced after treatment with GDNs (16.7 1/min without GDNs, 9.6 1/min after GDNs) (Table 5.18).

5.6.3. Glyceryl Di- and Mononitrate Pharmacokinetics

In two of the GTN infusions at 0.4 μg/min/kg, ^{3}H-GTN was employed to enable monitoring of the plasma concentration time profiles of GDNs and GMNs. Figure 5.44 and Tables A20 and A21 show that the ratio of formed 1,2-GDN to 1,3-GDN is approximately 2:1 in both femoral arterial and venous plasma samples. As found for GTN, an arterial-venous difference in plasma GDN concentrations was apparent. However, the difference is smaller than found for GTN (Figure 5.34). The arterial concentrations of GDN were greater than the venous concentrations during the infusion of GTN. On cessation of infusion, the arterial concentrations of GDN fell below the venous concentrations (Figure 5.44 and Tables A20 and A21). The average availabilities for 1,3-GDN and 1,2-GDN were 0.895 and 0.89, respectively (Table 5.19). The intrinsic clearance of the 1,3-GDN and 1,2-GDN (calculated using

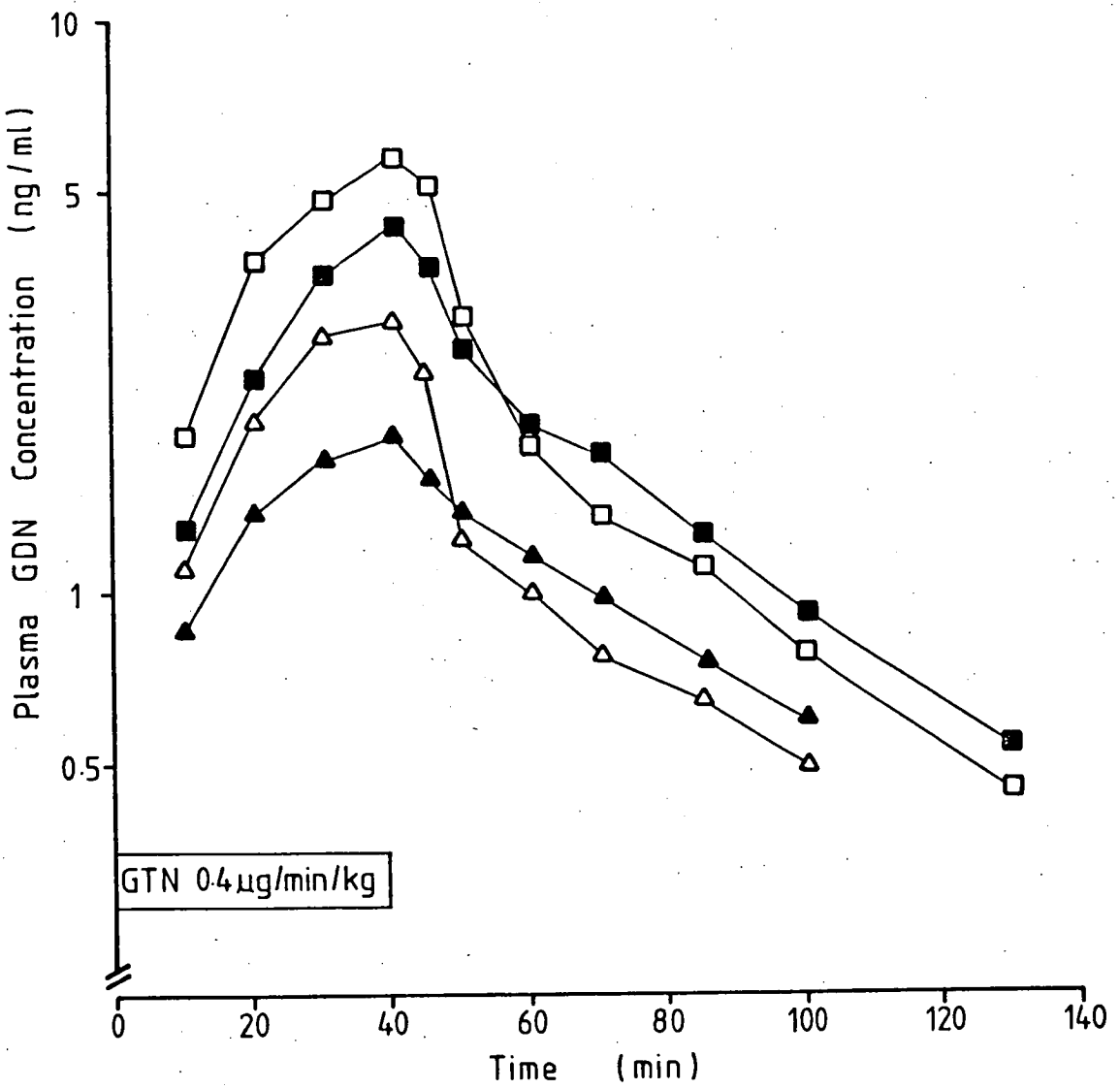


Figure 5.44 Time course of plasma 1,3-GDN (△ ▲) and 1,2-GDN (□ ■) concentrations in a femoral artery (open symbols) and the left femoral vein (closed symbols) of Sheep 16 receiving a continuous intravenous infusion of radio-labelled GTN.

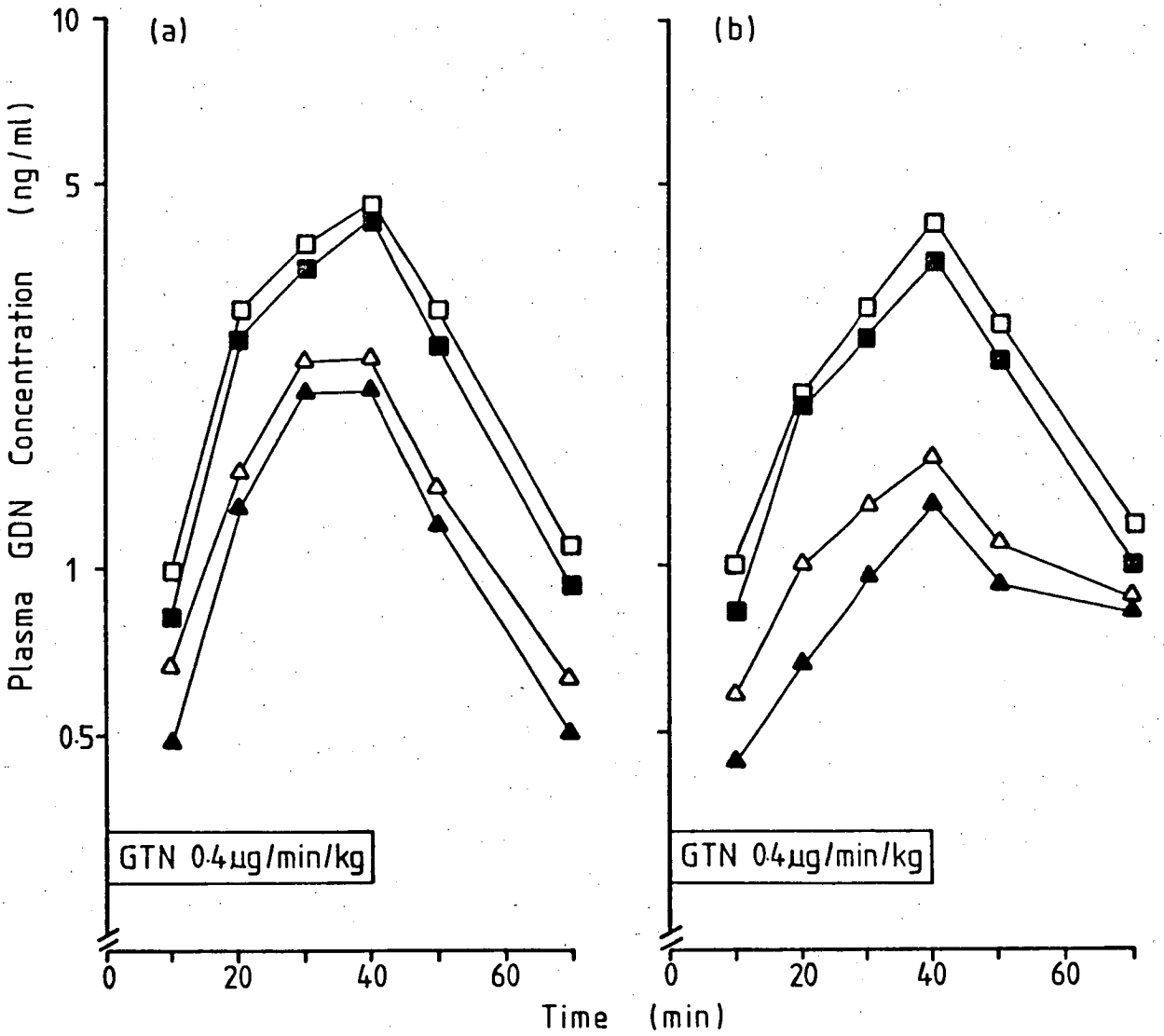


Figure 5.45 Time course of plasma 1,3-GDN (△ ▲) and 1,2-GDN (□ ■) concentrations in the portal vein (open symbols) and hepatic vein (closed symbols) of Sheep 16 (a) and Sheep 17 (b) receiving continuous intravenous infusions of radio-labelled GTN.

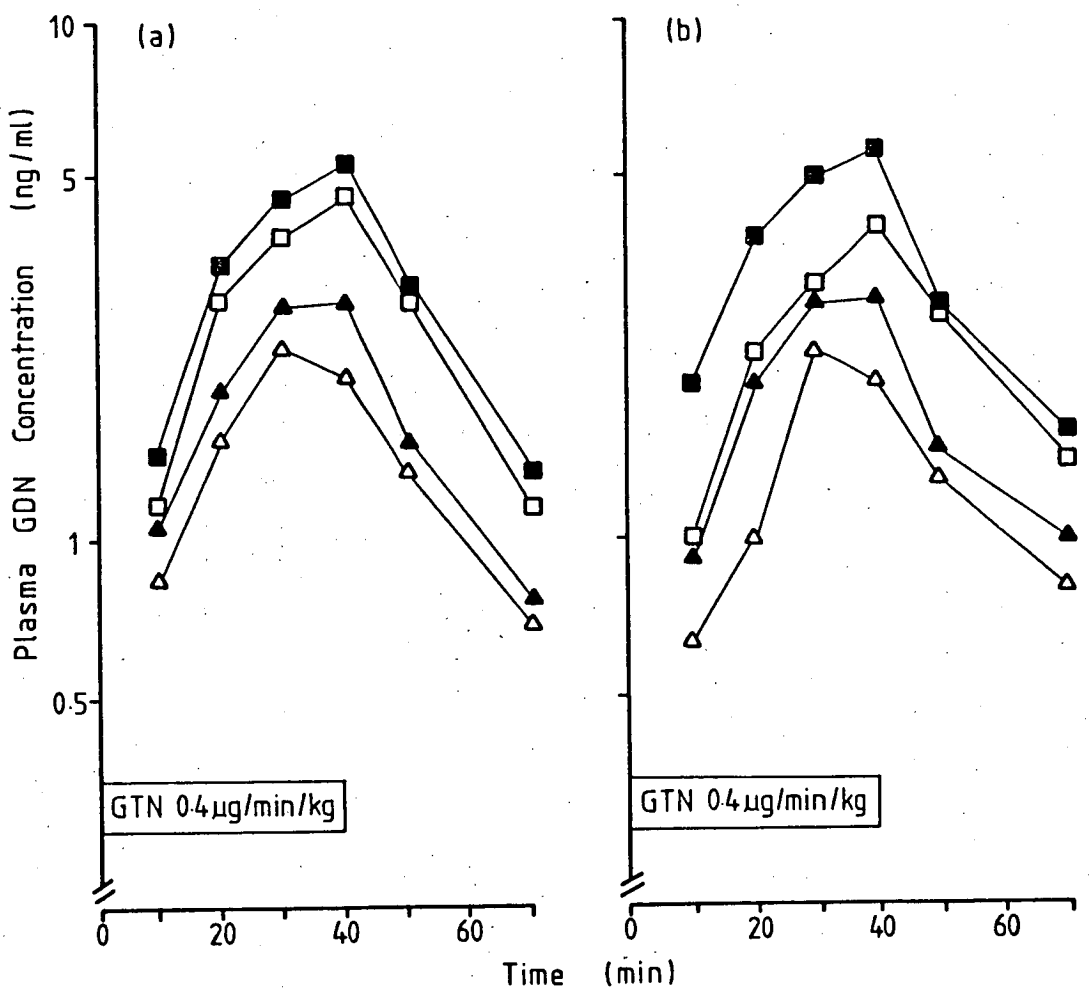


Figure 5.46 Time course of plasma 1,3-GDN (△ ▲) and 1,2-GDN (□ ■) concentrations in the pulmonary artery (open symbols) and left ventricle (closed symbols) of Sheep 16 (a) and Sheep 17 (b) receiving continuous intravenous infusions of radio-labelled GTN.

Table 5.19 Pharmacokinetic parameters of glyceryl dinitrates (GDN) formed during nitroglycerin infusions (0.4 µg/min/kg) in 2 sheep.

Drug	Availability			Intrinsic Clearance (l/min)			Mean Residence Time (min)			Terminal Half-life[a] (min)
	leg	lung	liver	leg	lung	liver	leg	lung	liver	
1,3-GDN	0.09	1.20	0.83	0.024	-	0.109	12.9	0	0	43.5
	0.89	1.33	0.86	0.034	-	0.117	11.3	0	5.2	39.7
1,2-GDN	0.91	1.15	0.88	0.024	-	0.084	8.0	0	0	35.0
	0.87	1.29	0.87	0.048	-	0.123	9.2	0	0	34.5

[a]Calculated from the terminal phase of log GDN venous plasma concentration-time profiles.

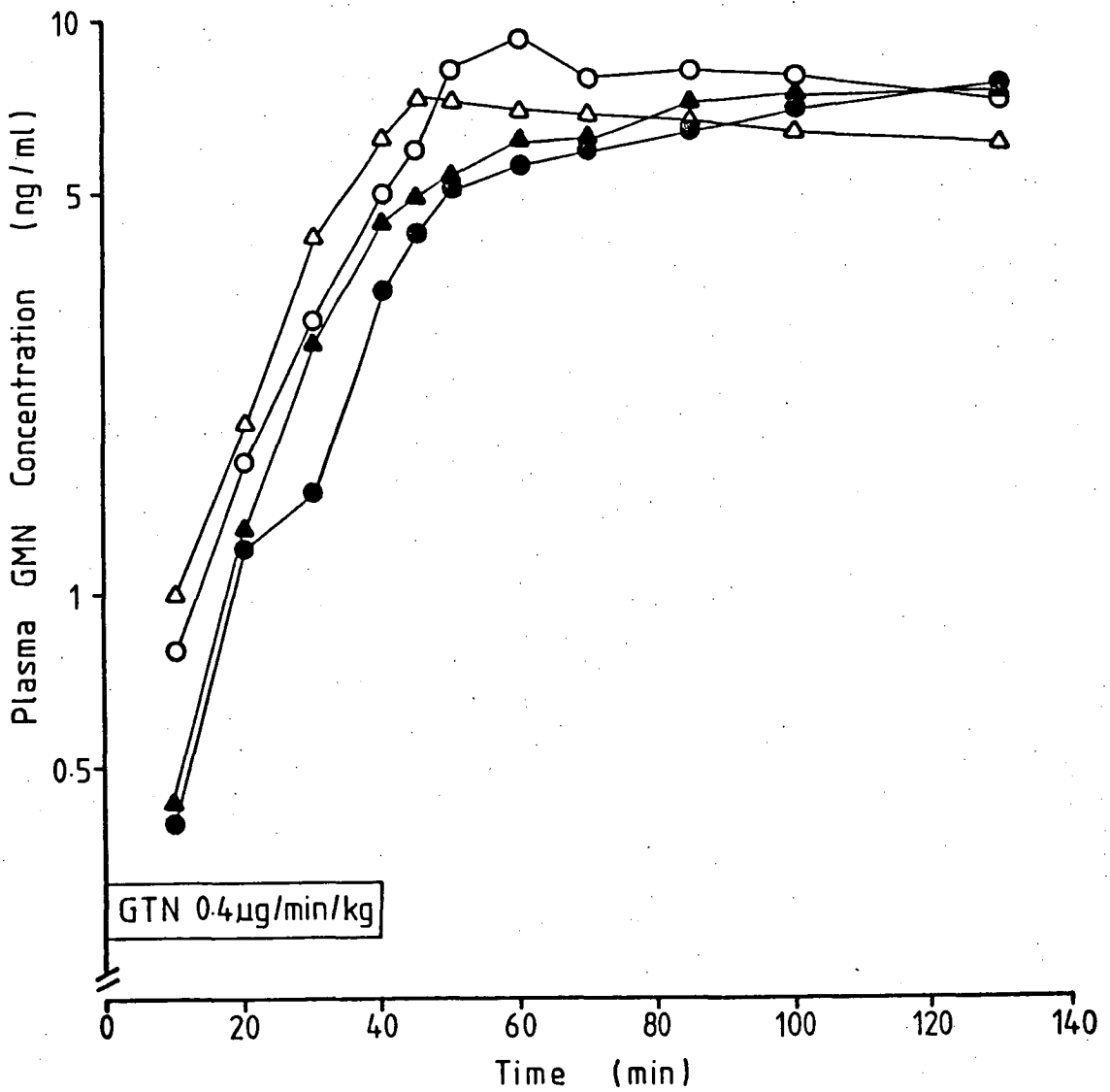


Figure 5.47 Time course of plasma GMN concentrations in a femoral artery (open symbols) and the left ventricle (closed symbols) of Sheep 16 (△ ▲) and Sheep 17 (○ ●) receiving continuous intravenous infusions of radio-labelled GTN.

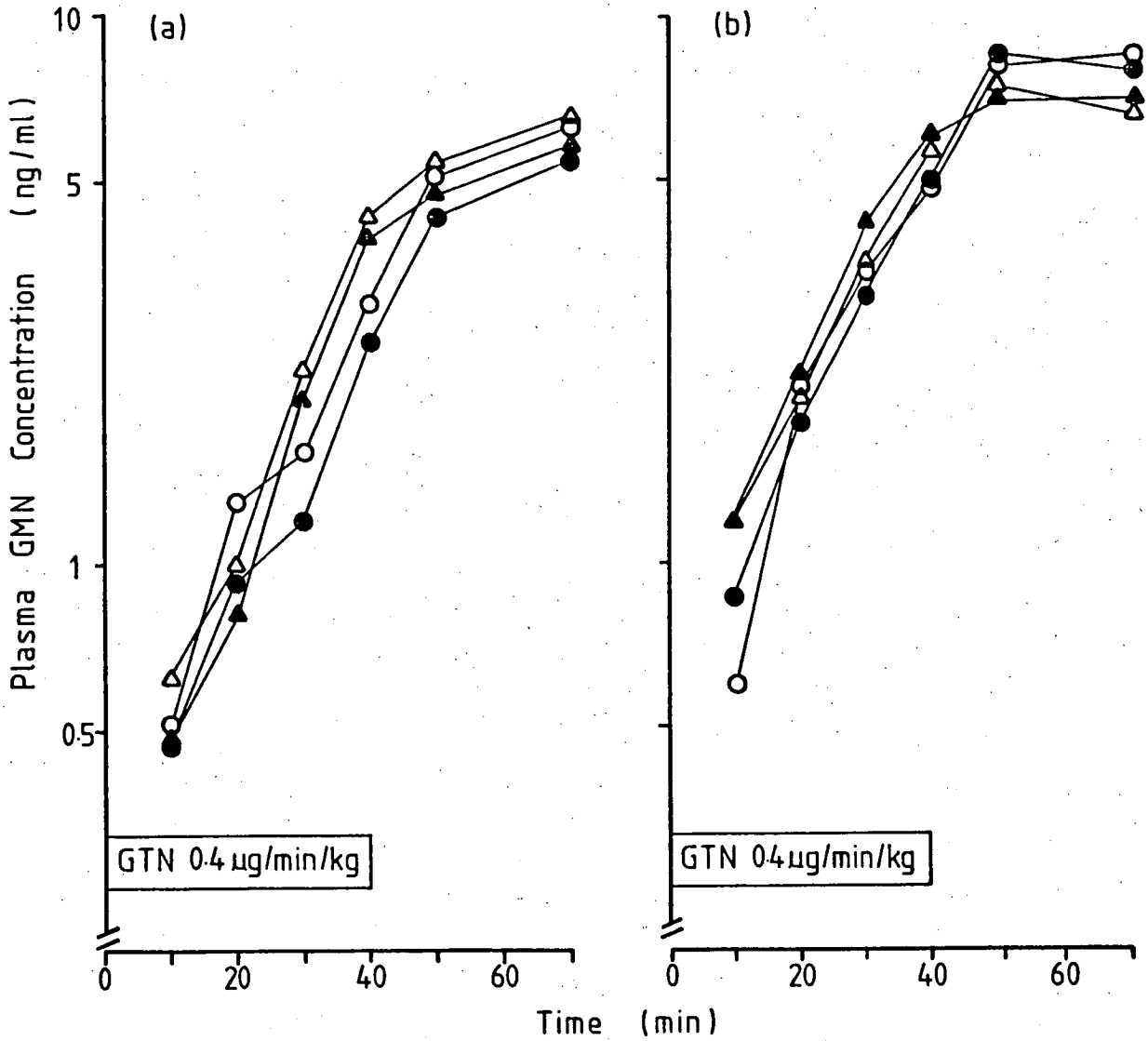


Figure 5.48 Time course of plasma GMN concentrations in blood entering and leaving (a) the liver (open symbols, portal vein; closed symbols, hepatic vein) and (b) the lungs (open symbols, pulmonary artery; closed symbols, left ventricle) of Sheep 16 (△ ▲) and Sheep 17 (○ ●) receiving continuous intravenous infusions of radio-labelled GTN.

f_u values of 0.89 and 0.77 respectively) averaged 0.029 and 0.036 l/min respectively. Mean residence times in the leg for 1,3-GDN and 1,2-GDN are 12.1 min and 8.6 min, respectively (Table 5.19). The half-lives of the terminal phase of the decay of plasma 1,3-GDN and 1,2-GDN plasma concentrations are about 35 min and 39 min respectively (Figure 5.44).

Figures 5.45 and 5.46 and Tables A20 and A21 show plasma GDN concentrations in blood entering and leaving the liver and lungs. The concentrations of GDN in blood leaving the lungs (left ventricle) exceed that in the pulmonary artery (Figure 5.46) giving availabilities for the GDNs across the lungs greater than unity (Table 5.19). As GMNs continued to be formed at the conclusion of each study, it was not possible to obtain AUCs and therefore availabilities for those compounds. Figure 5.47 and Tables A22 and A23 show that an arteral-venous difference in plasma GMN concentrations exists across the leg during the infusion of GTN. This difference gradually decreases as the plasma concentrations of GMNs increase and eventually at about 80-120 min the concentration of GMNs in venous plasma exceeds that in the arterial plasma (Figure 5.47). Across the liver, the portal vein GMN concentrations exceeded the hepatic vein GMN concentrations at all times studied (Figure 5.48 (a)). The concentrations of GMNs in the pulmonary artery and left ventricle fluctuated but were similar over the time of blood sampling (Figure 5.48 (b)).

The total radioactivity recovered in the fractions of the HPLC effluent corresponding to GTN, GDNs and GMNs accounted for more than 96% of the total radioactivity in plasma injected into the HPLC column, indicating that little, if any, of the label was present as glycerol or a conjugated metabolite.

5.7. SALICYLATE PHARMACOKINETICS AFTER ASPIRIN INFUSIONS IN SHEEP

5.7.1. Aspirin, Salicylic Acid and Salicyluric Acid Pharmacokinetics

Plasma concentrations of total ASA and SA in arterial and venous samples from four sheep receiving 485 μg ASA/min/kg are shown in Figure 5.49 and Tables A24 to A27. An arterio-venous difference exists for both ASA and SA during the ASA infusion but is more pronounced for ASA than for SA. At later times after the termination of the infusion the venous levels of ASA and SA are slightly greater than the arterial levels (Figure 5.49). Figure 5.50 and Tables A28 to A31 show corresponding free ASA and SA concentrations and f_u in two of the same four sheep. It can be seen that the f_u of ASA is 0.55-0.75 while that of SA is about 0.3. Total and free SU levels for the same two sheep are shown in Figure 5.51 a, b and Table A32. The unbound fraction of SU in venous and arterial plasma is plotted against time in Figure 5.52 (a,b) and is seen to be 0.30-0.35 for the two sheep studied.

Plasma total ASA and SA concentrations in blood samples taken from portal and hepatic veins (i.e., across the liver) for the four sheep receiving 485 μg ASA/min/kg are presented in Figure 5.53 and Tables A24 to A27. At all times studied the ASA and SA concentrations in the portal blood exceeded that in the hepatic vein (Figure 5.53). In contrast, SU concentrations (Figure 5.54 (a) and (b) and Table A33) were always greater in the hepatic vein blood than in the portal vein blood.

Figure 5.55 (and Tables A24 to A27) shows that the concentrations of ASA and SA across the lungs were often greater in venous blood than in arterial blood both during and after infusion of ASA.

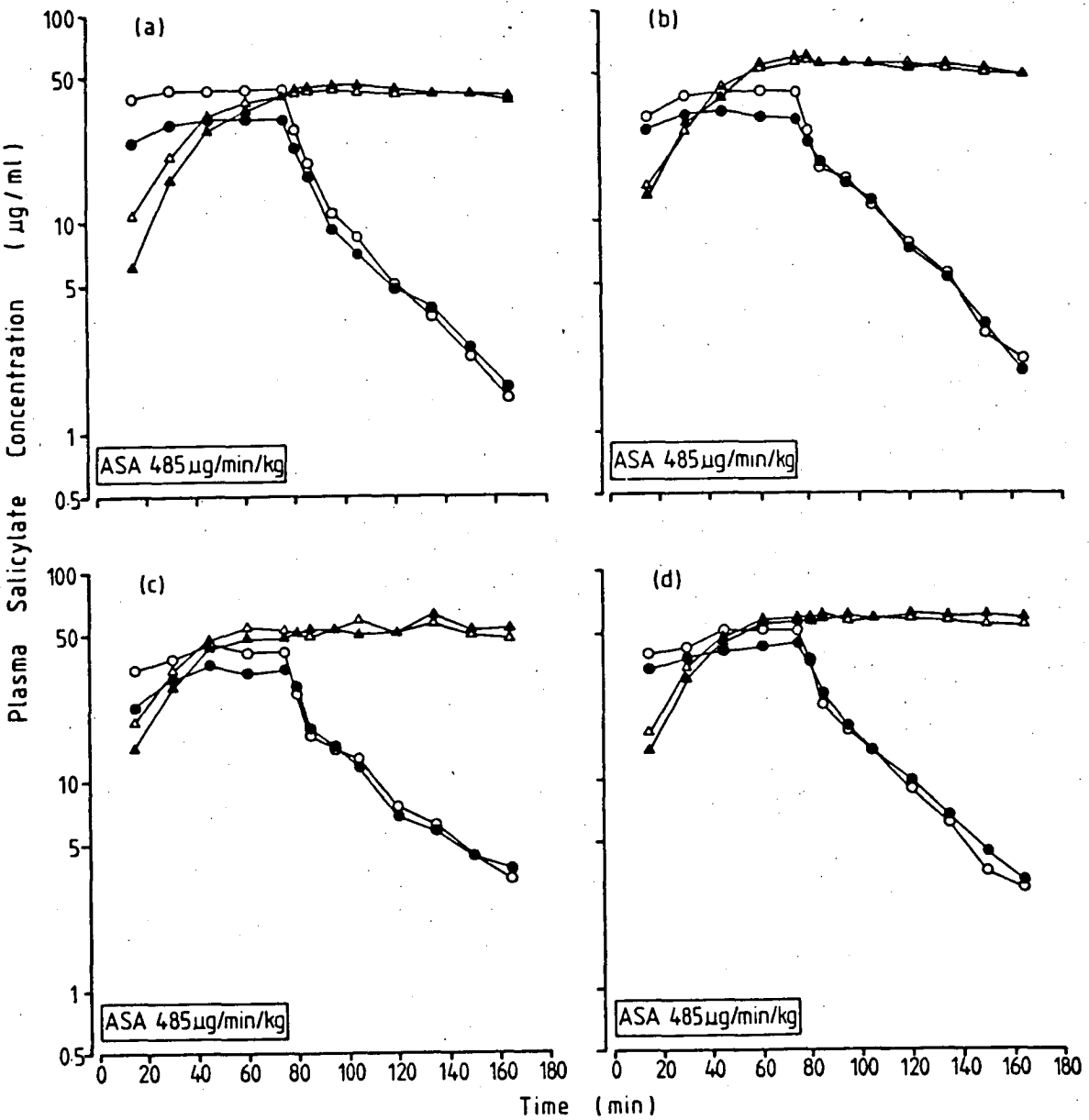


Figure 5.49 Femoral arterial (open symbols) and venous (closed symbols) plasma ASA (○ ●) and SA (△ ▲) total concentration-time profiles for Sheep 23,(a); Sheep 24, (b); Sheep 25, (c); Sheep 28, (d) receiving continuous intravenous infusions of ASA.

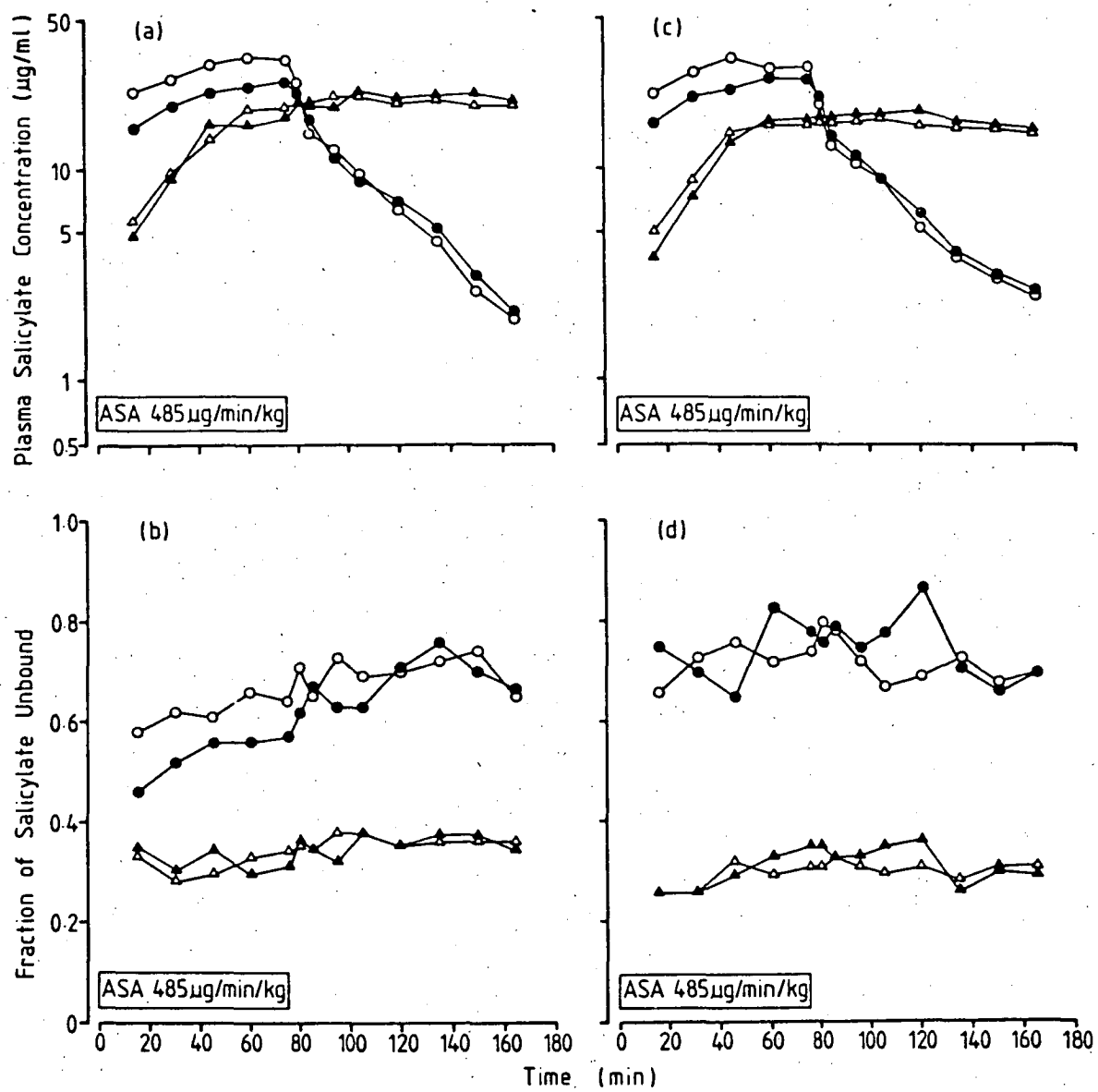


Figure 5.50 Femoral arterial (open symbols) and venous (closed symbols) plasma ASA (○ ●) and SA (△ ▲) free concentration-time profiles and free fraction-time profiles in 2 sheep receiving continuous intravenous ASA infusions. Sheep 25, (a) (b); Sheep 28, (c) (d).

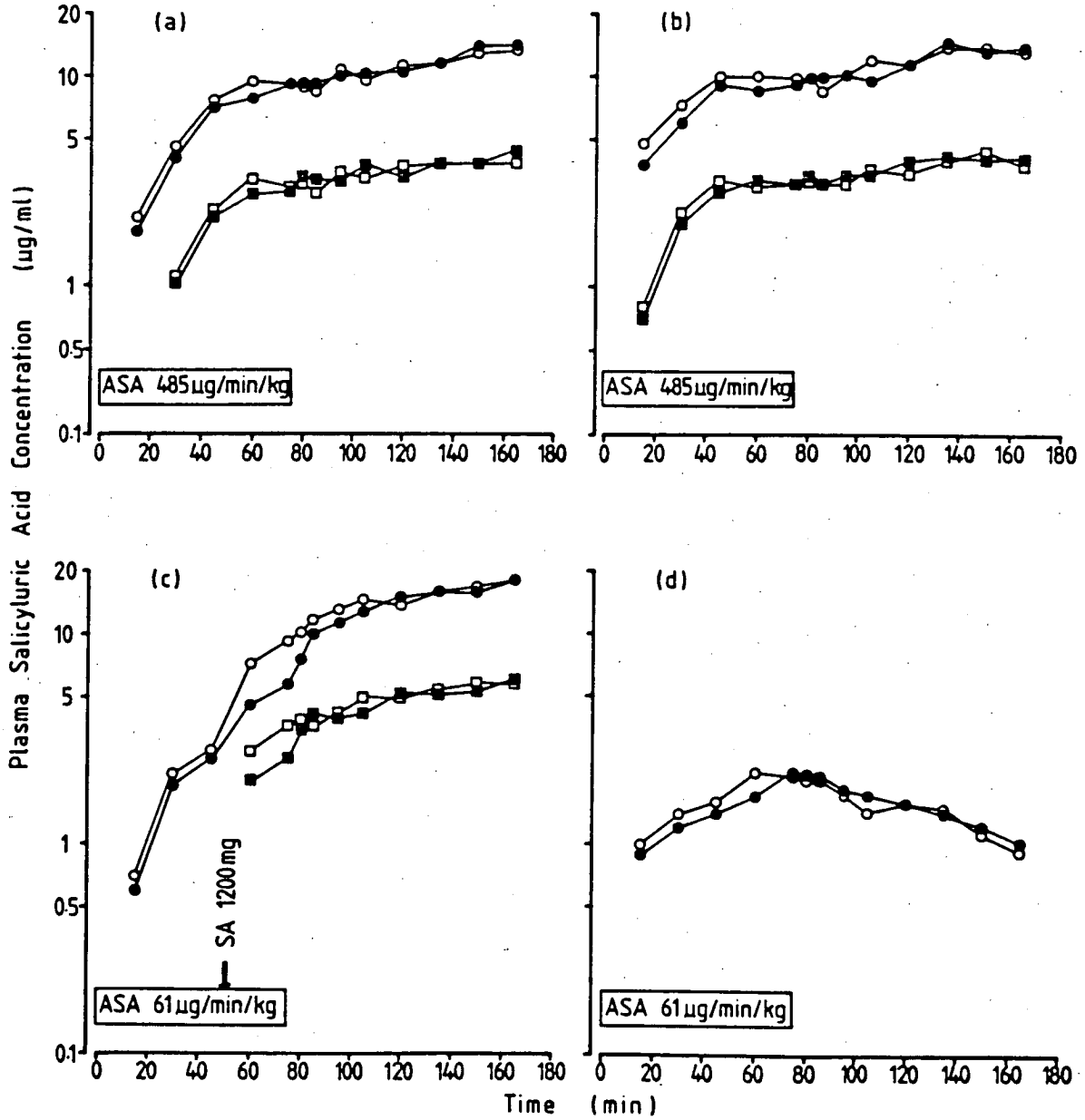


Figure 5.51 Femoral arterial (open symbols) and venous (closed symbols) plasma total (○ ●) and free (□ ■) SU concentration-time profiles in sheep receiving continuous intravenous ASA infusions.
Sheep 25, (a); Sheep 28, (b); Sheep 29, (c); Sheep 30, (d).

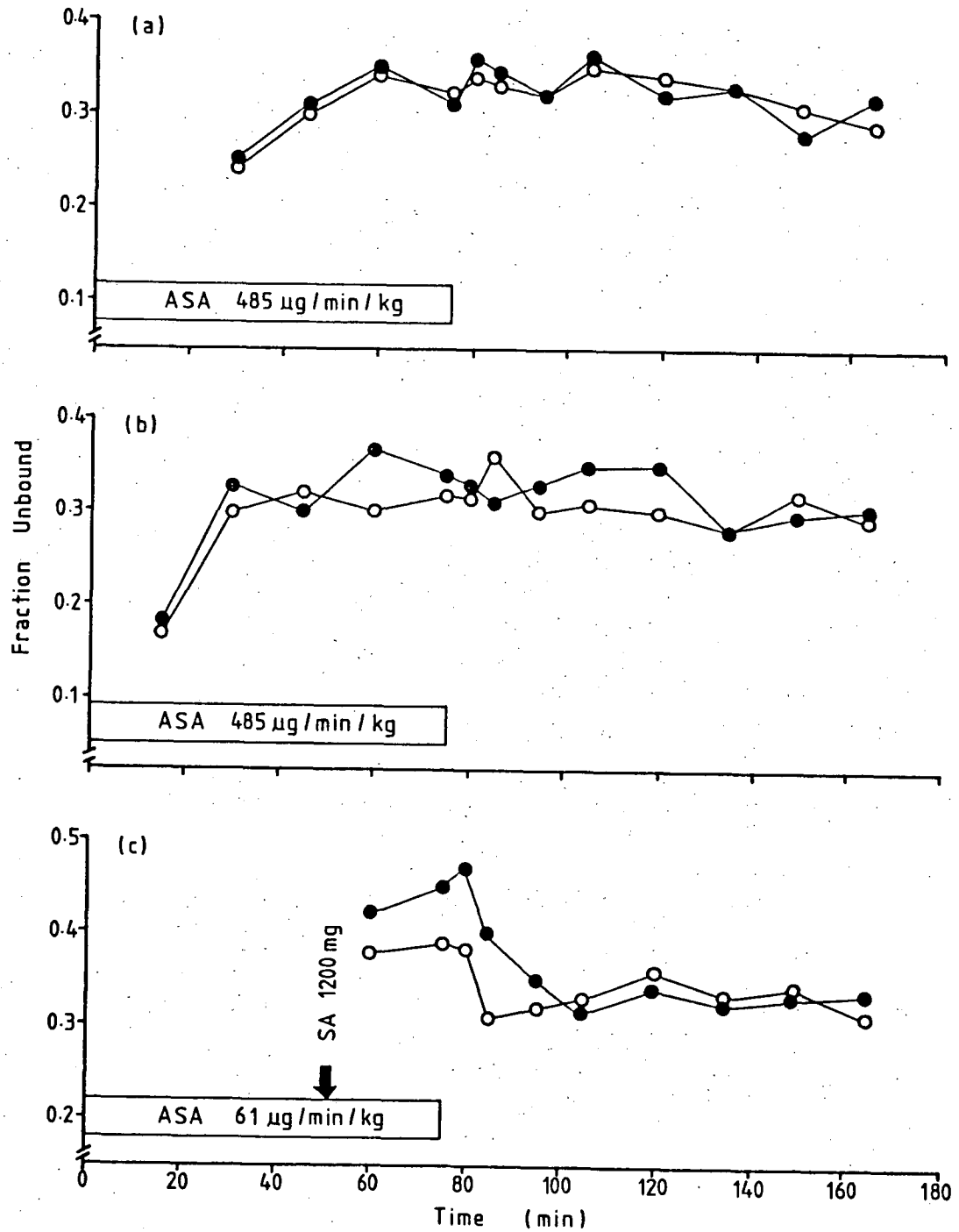


Figure 5.52 The time course of the free fraction of SU in femoral arterial (open symbols) and venous (closed symbols) plasma during continuous intravenous infusions of ASA in Sheep 25, (a); Sheep 28, (b) and Sheep 29, (c).

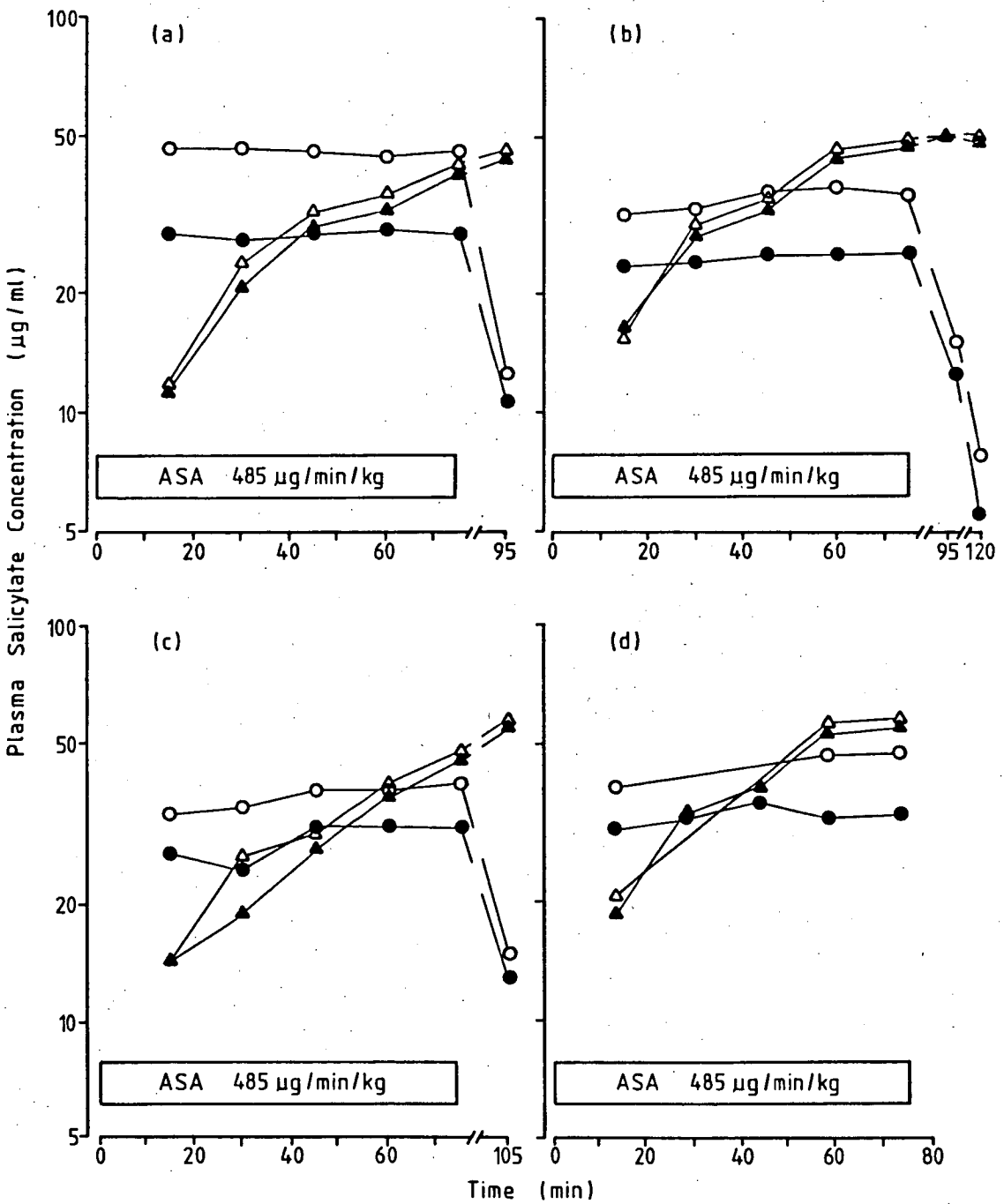


Figure 5.53 Portal vein (open symbols) and hepatic vein (closed symbols) plasma ASA (○ ●) and SA (△ ▲) total concentration-time profiles for Sheep 23, (a); Sheep 24, (b); Sheep 25, (c); Sheep 28, (d) receiving continuous intravenous infusions of ASA.

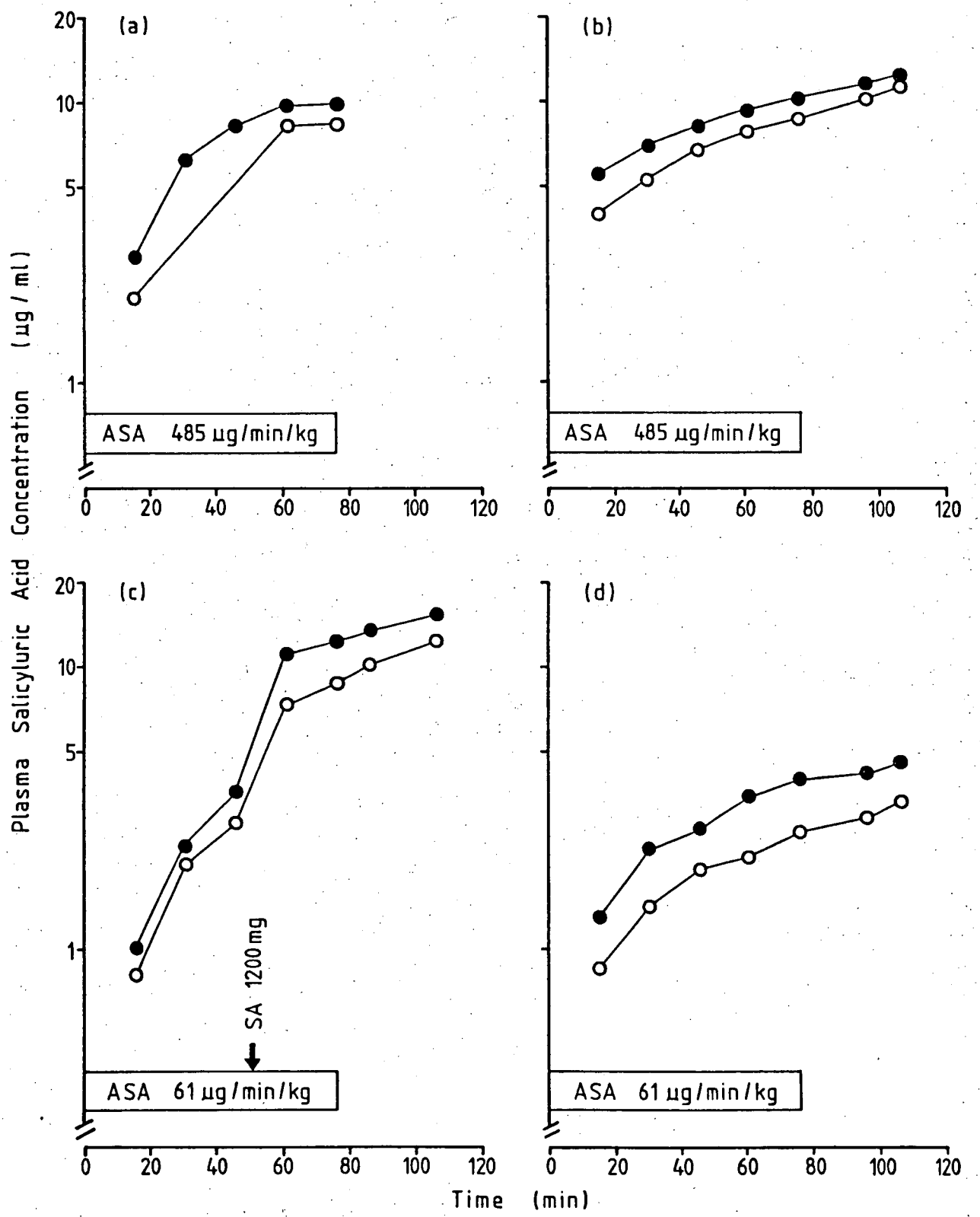


Figure 5.54 Portal vein (open symbols) and hepatic vein (closed symbols) plasma SU concentration-time profiles for Sheep 25, (a); Sheep 28, (b); Sheep 29, (c) and Sheep 30, (d) receiving continuous intravenous infusions of ASA.

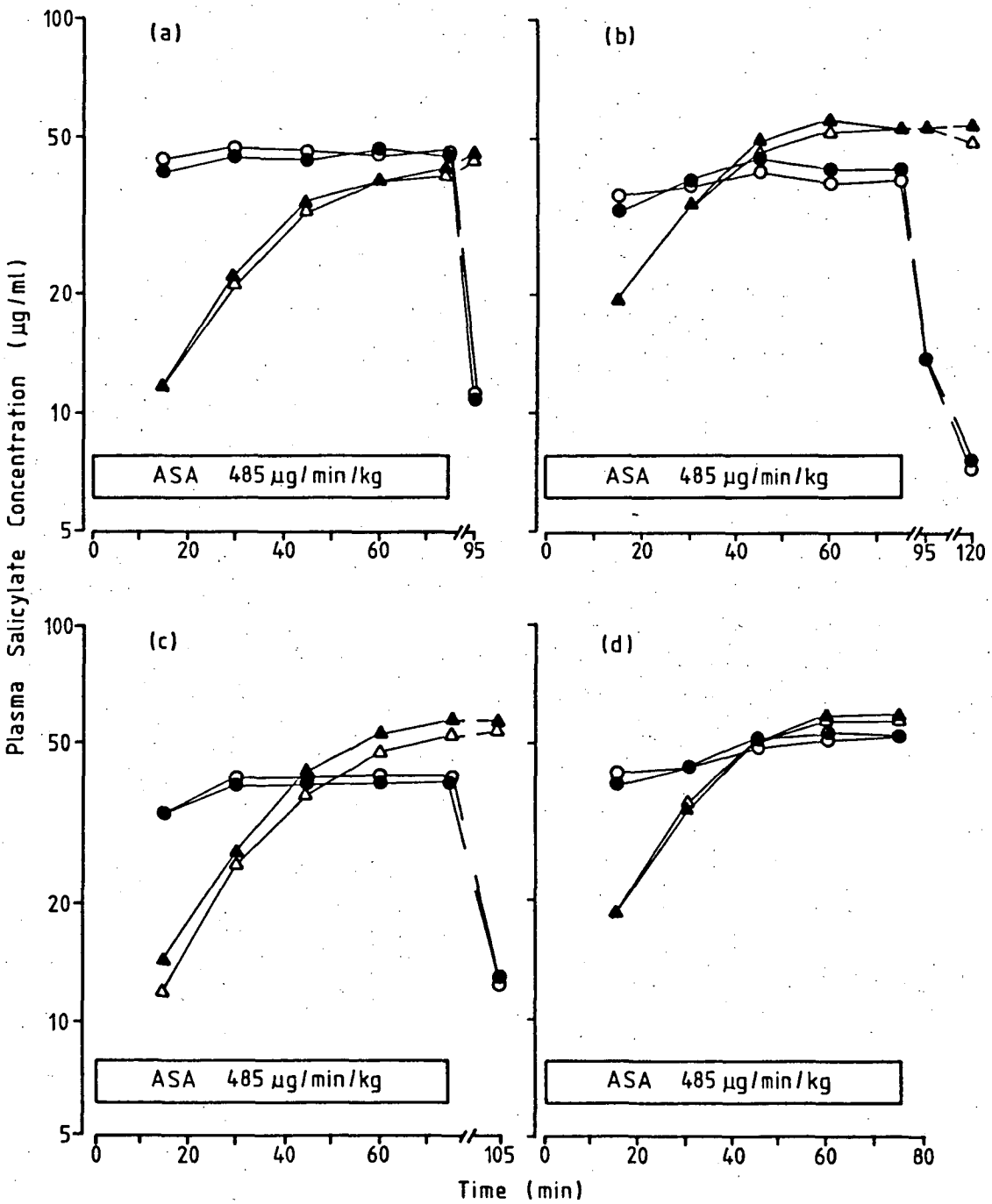


Figure 5.55 Pulmonary artery (open symbols) and left ventricle (closed symbols) plasma ASA (○ ●) and SA (△ ▲) concentration-time profiles for Sheep 23, (a); Sheep 24, (b); Sheep 25, (c) and Sheep 28, (d) receiving continuous intravenous infusions of ASA.

5.7.2. Effect of Salicylic Acid on Aspirin Pharmacokinetics

Figures 5.56 and 5.57 and Tables A34 to A45 show plasma levels of total and free ASA and SA in the arterial and venous blood of four sheep receiving 61 µg ASA/min/kg. In three of the sheep (Figures 5.56 and 5.57 (b), (c), (d)), bolus doses (1 per sheep) of 30, 300 and 1200 mg SA were given at the 50th minute of ASA infusion. Addition of SA appeared to have no effect on the magnitude of the arterial-venous differences in total and free ASA concentrations in any of the animals. However, the rate of elimination of ASA on termination of the ASA infusion was faster in the sheep receiving the 1200 mg SA bolus dose (half-life of elimination approximately 17 min for total ASA concentrations) than in the other sheep (half-lives of elimination approximately 22 to 32 min for total ASA concentrations). In the sheep receiving 1200 mg SA the plasma protein binding of ASA changed on administration of the SA (Figure 5.58 (d)), whereas the fraction of salicylate unbound in the other sheep was not noticeably affected by the administration of smaller bolus doses of SA (Figure 5.58 (b), (c)).

The elimination of SA in the sheep that received the 1200 mg bolus dose of SA was slower than in the other three sheep (Figures 5.56 and 5.57 (a), (b), (c), (d)).

Total and free SU concentrations across the hind-leg of two sheep receiving ASA infusions at 61 µg/min/kg are shown in Figure 5.51 (c), (d) and Table A32. An apparent initial arterial-venous difference in SU plasma concentrations was diminished with time. Figure 5.52 (c) shows that a 1200 mg bolus dose of SA probably increased the unbound fraction of SU temporarily, however, this interpretation is made

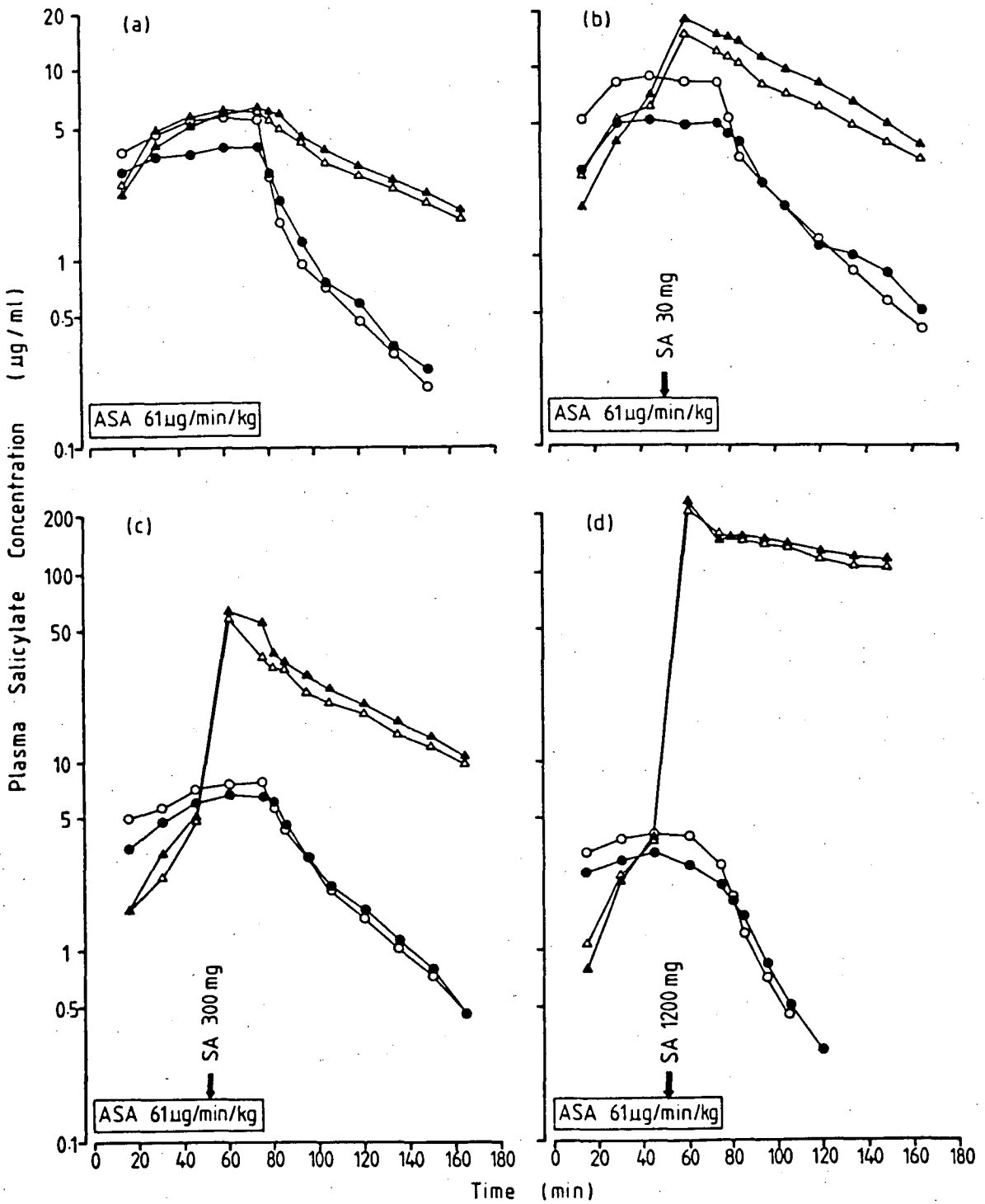


Figure 5.56 Femoral arterial (open symbols) and venous (closed symbols) plasma ASA (○ ●) and SA (△ ▲) total concentration-time profiles for Sheep 30, (a); Sheep 27, (b); Sheep 26, (c) and Sheep 29, (d) receiving a continuous intravenous infusion of ASA with or without a bolus dose of SA.

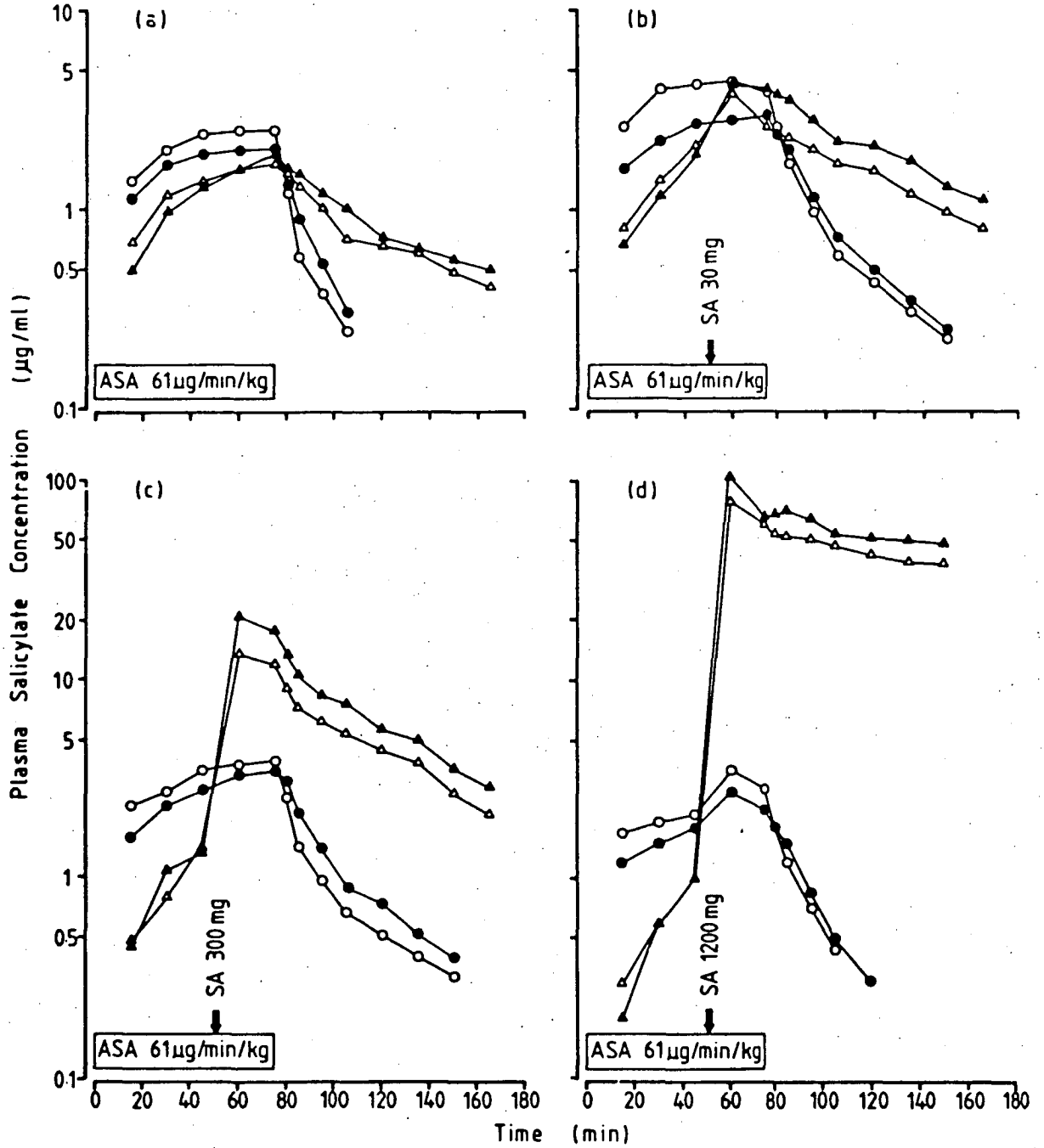


Figure 5.57 Femoral arterial (open symbols) and venous (closed symbols) plasma ASA (○ ●) and SA (△ ▲) free concentration-time profiles for Sheep 30, (a); Sheep 27, (b); Sheep 26, (c) and Sheep 29, (d) receiving a continuous intravenous infusion of ASA with or without a bolus dose of SA.

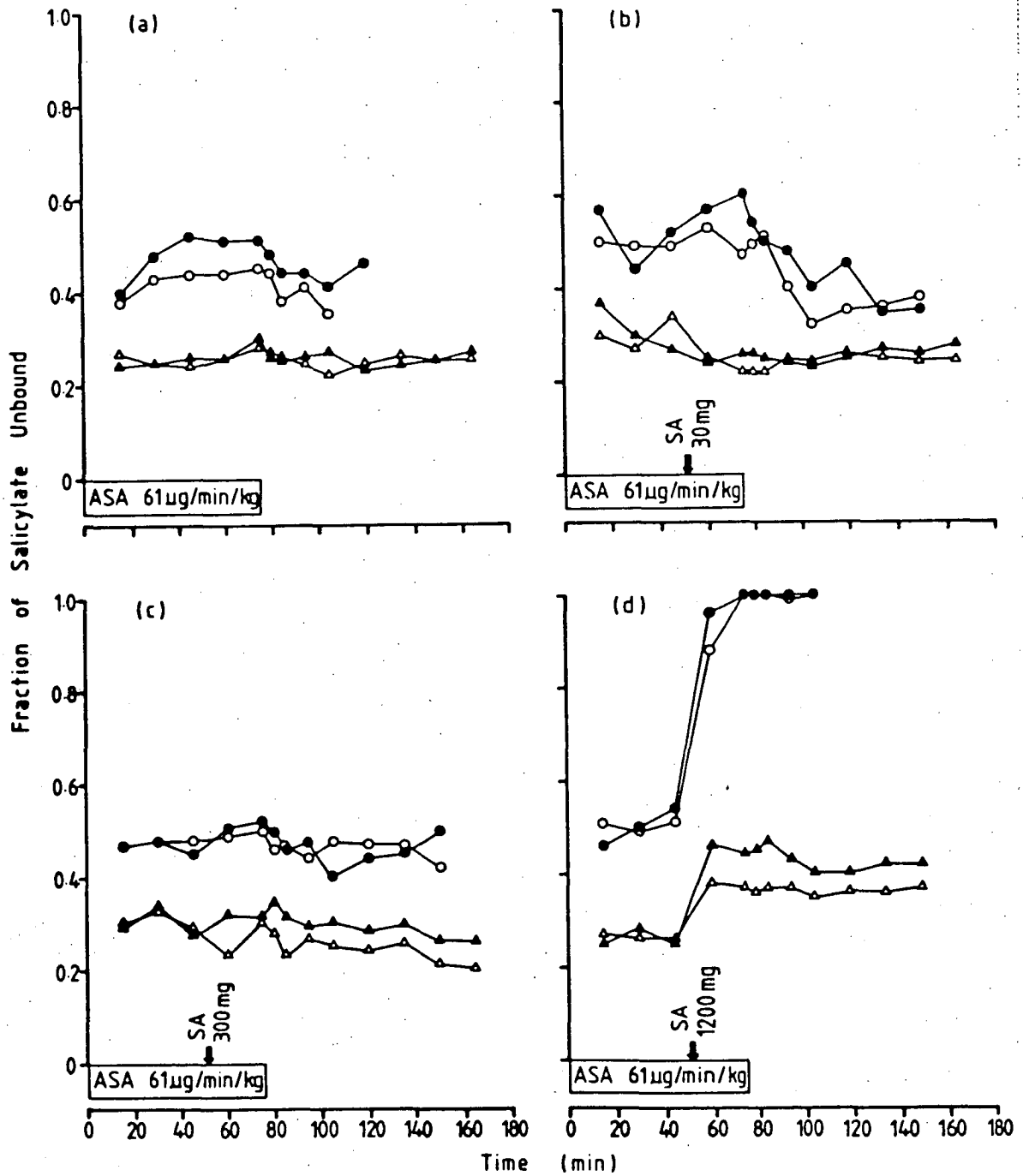


Figure 5.58 The time course of the free fraction of ASA (○ ●) and SA (△ ▲) in femoral arterial (open symbols) and venous (closed symbols) plasma of Sheep 30, (a); Sheep 27, (b); Sheep 26, (c) and Sheep 29, (d) receiving continuous intravenous infusions of ASA with and without a bolus dose of SA.

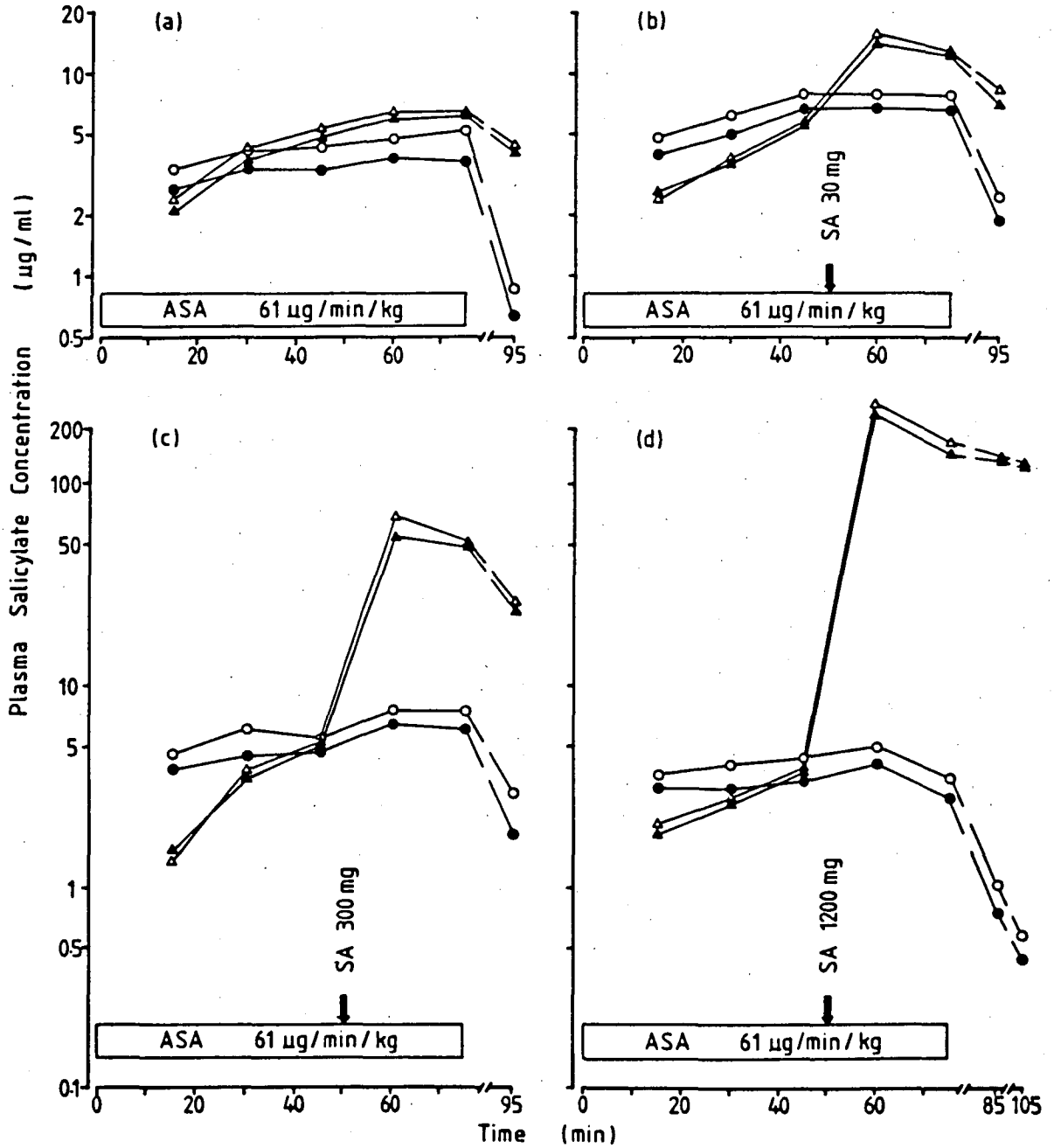


Figure 5.59 Portal vein (open symbols) and hepatic vein (closed symbols) plasma ASA (○ ●) and SA (△ ▲) total concentration-time profiles in Sheep 30, (a); Sheep 27, (b); Sheep 26, (c) and Sheep 29, (d) receiving continuous intravenous infusions of ASA with or without a bolus dose of SA.

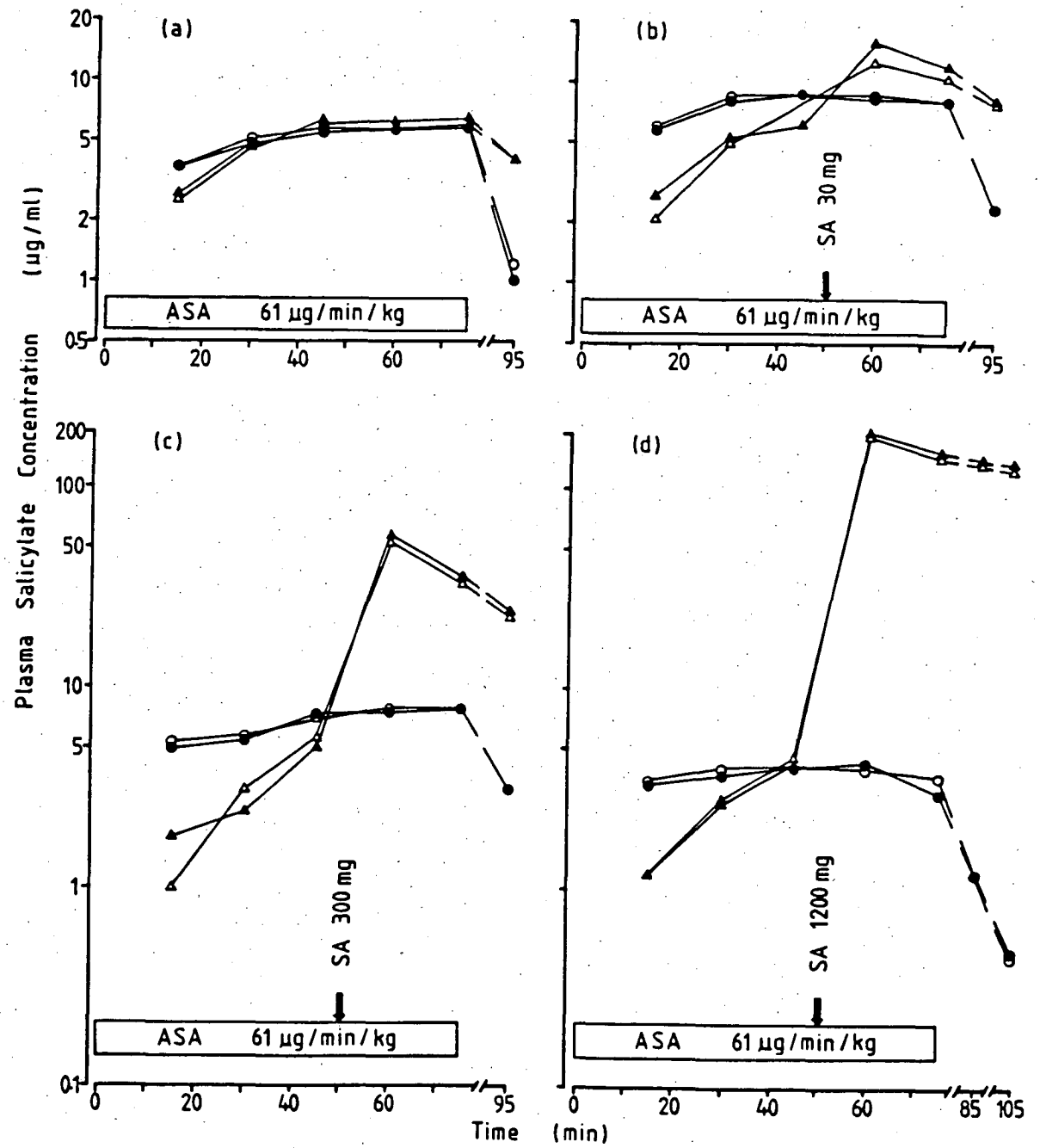


Figure 5.60 Pulmonary artery (open symbols) and left ventricle (closed symbols) plasma ASA (○ ●) and SA (△ ▲) total concentration-time profiles in Sheep 30, (a); Sheep 27, (b); Sheep 26, (c) and Sheep 29, (d) receiving continuous intravenous infusions of ASA with or without a bolus dose of SA.

Table 5.20 Comparison of aspirin pharmacokinetic parameters using total and free aspirin plasma concentrations.

Infusion Rate	Bolus Dose SA	Total ASA			Free ASA		
		Availability across leg	Intrinsic clearance across leg	Mean Residence Time	Availability across leg	Intrinsic Clearance across leg	Mean Residence Time
(μg/min/kg)	(mg)		(l/min)	(min)		(l/min)	(min)
61	0	0.79	0.135	4.4	0.88	0.072	4.2
61	30	0.69	0.257	9.6	0.73	0.214	8.7
61	300	0.92	0.054	2.9	0.95	0.032	7.5
61	1200	0.82	0.050	3.0	0.86	0.037	4.1
485	0	0.91	0.027	3.1	0.81	0.063	5.8
485	0	0.85	0.053	5.2	0.81	0.070	9.7

Table 5.21 Pharmacokinetic parameters of aspirin (ASA) after continuous intravenous infusions in sheep. (Mean ± SE, n=4).

ASA Infusion Rate (µg/min/kg)	Availability			Intrinsic Clearance (l/min)			Mean Residence Time (min)		
	leg	lung	liver	leg	lung	liver	leg	lung	liver
61	0.80 ± 0.06	0.96 ± 0.03	0.77 ± 0.01	0.12 ± 0.06	0.16 ± 0.06	0.35 ± 0.08	4.97 ± 1.86	0.72 ± 0.85	1.32 ± 0.90
485	0.86 ± 0.06	1.00 ± 0.01	0.72 ± 0.03	0.05 ± 0.02	0.02 ± 0.02	0.28 ± 0.04	3.71 ± 1.01	0.37 ± 0.20	1.83 ± 1.29

Table 5.22 Individual systemic clearances (calculated as dose/AUC) and terminal half-lives of aspirin (ASA) after continuous intravenous infusions in sheep.

ASA Infusion Rate (µg/min/kg)	Salicylic Acid Bolus Dose (mg)	Systemic Arterial Clearance (l/min)	Systemic Venous Clearance (l/min)	Terminal Half-life[a] (min)
61	0	0.32	0.41	27.2
	30	0.18	0.26	31.5
	300	0.22	0.24	22.5
	1200	0.44	0.54	16.8
485	0	0.30	0.42	28.5
	0	0.23	0.24	22.5
	0	0.20	0.22	26.2
	0	0.25	0.29	32.5

[a] Calculated from the terminal phase of log total ASA venous plasma concentration-time profiles.

Table 5.23 Pharmacokinetic parameters of salicylic acid (SA) formed during aspirin (ASA) infusions at 61 µg/min/kg in 3[a] sheep. (Mean ± S.E.)

ASA Infusion Rate (µg/min/kg)	Availability			Intrinsic Clearance (l/min)			Mean Residence Time (min)		
	leg	lung	liver	leg	lung	liver	leg	lung	liver
61	1.19 ± 0.02	1.05 ± 0.02	0.91 ± 0.01	-	-	0.25 ± 0.04	0.73 ± 0.76	0.17 ± 0.17	0.30 ± 0.30

[a] AUCs for SA in the sheep receiving a bolus dose of 1200 mg SA were not calculated because of the non-linear elimination kinetics of SA at the plasma concentrations found in this study.

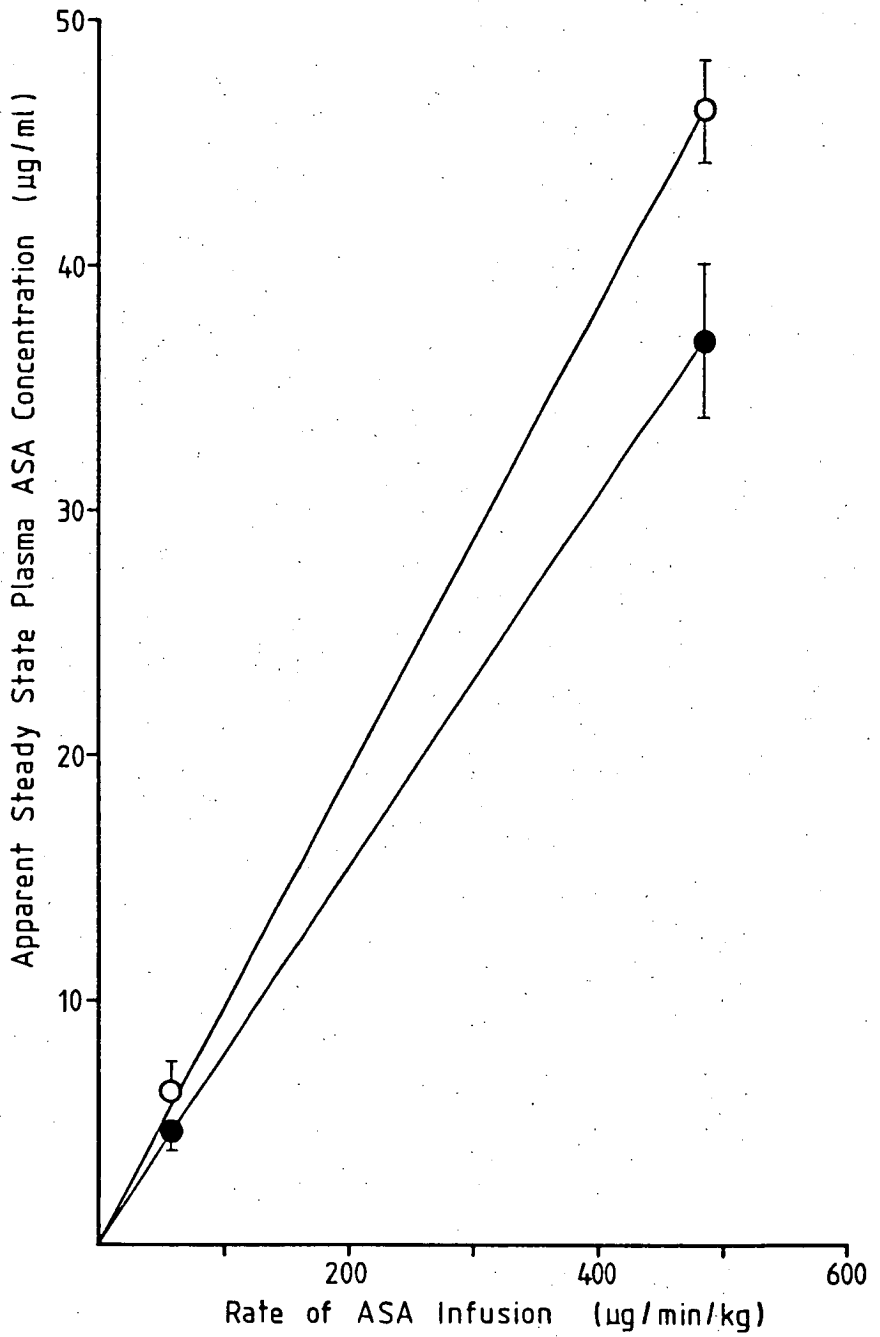


Figure 5.61 Apparent steady state femoral arterial (open symbols) and venous (closed symbols) plasma total ASA concentrations resulting from continuous intravenous infusions of ASA in sheep. Each point represents the mean ± se of the results for 4 sheep.

difficult by the fact that preceding the SA bolus dose, free SU concentrations were undetectable.

Figure 5.59 (a) shows that the plasma concentrations of ASA and SA in the hepatic vein were less than those in the portal vein during and after ASA infusions. This difference was maintained after bolus doses of SA (Figure 5.59 (b), (c), (d) and Tables A37, A40 and A43). In contrast, Figure 5.60 and Tables A34, A37, A40 and A43 show no differences in ASA plasma concentrations in the pulmonary artery and left ventricle before or after bolus doses of SA. However, following SA bolus doses there was a consistent difference in plasma SA concentrations, those in the left ventricle being greater than those in the pulmonary artery.

Plasma SU concentrations were always greater in the hepatic vein than in the portal vein during ASA infusions of 61 µg/min/kg and this difference was unaffected by a 1200 mg bolus dose of SA (Figure 5.54 (c), (d) and Table A33).

5.7.3. Salicylate Kinetics in Relation to Dose

For the two ASA infusion rates studied, apparent steady-state plasma ASA concentrations were generally achieved within 60 min of the commencement of ASA infusion. A linear relationship appears to exist between the rate of infusion of ASA and its apparent steady-state concentration in femoral venous and arterial plasma (Figure 5.61). Tables 5.20, 5.21 and 5.22 show that the total clearance and other pharmacokinetic data derived for ASA appear to be independent of ASA dose or presence of added SA. Some pharmacokinetic parameters derived for SA are presented in Table 5.23. These data were derived only for ASA infusions of 61 µg/min/kg because the SA formed upon ASA infusions

at a rate of 485 µg/min/kg had only just reached a plateau by the end of the blood sampling times.

5.8 PHARMACOKINETICS AND PHARMACODYNAMICS OF SALICYLATES IN MAN

5.8.1. Single Dose Study

Plasma ASA and SA Concentrations

Figures 5.62 (a) and 5.63 (a) show the mean ASA and SA concentrations after ingestion of the soluble and the slow-release ASA. Figure 5.64 (a) shows the mean SA concentrations after ingestion of an equivalent dose of sodium salicylate. ASA was absorbed much more slowly from the slow-release formulation than from the soluble product which is evident from the longer peak concentration times and lower plasma ASA concentrations for the slow-release product. The times to reach peak ASA and SA concentrations were 3.33 ± 0.55 and 4.38 ± 0.29 hr for the slow-release formulation and 0.38 ± 0.04 and 0.91 ± 0.16 hr for the soluble formulation, respectively. The mean peak ASA concentration for the slow-release product (0.61 ± 0.22 µg/ml) was one-sixth that achieved for the soluble form (3.65 ± 0.81 µg/ml). In two subjects taking the slow-release preparation, no ASA was detectable (<0.1 µg/ml) in the plasma.

The area under the plasma ASA concentration-time curve was less for the slow-release formulation (0.79 ± 0.26 µg.hr/ml) than for the soluble product (2.84 ± 0.40 µg.hr/ml). However, the differences in area do not result from differences in absorption as the area under the metabolite (salicylate) concentration-time curve was similar for the slow-release (39.45 ± 4.0 µg.hr/ml) and soluble (33.37 ±

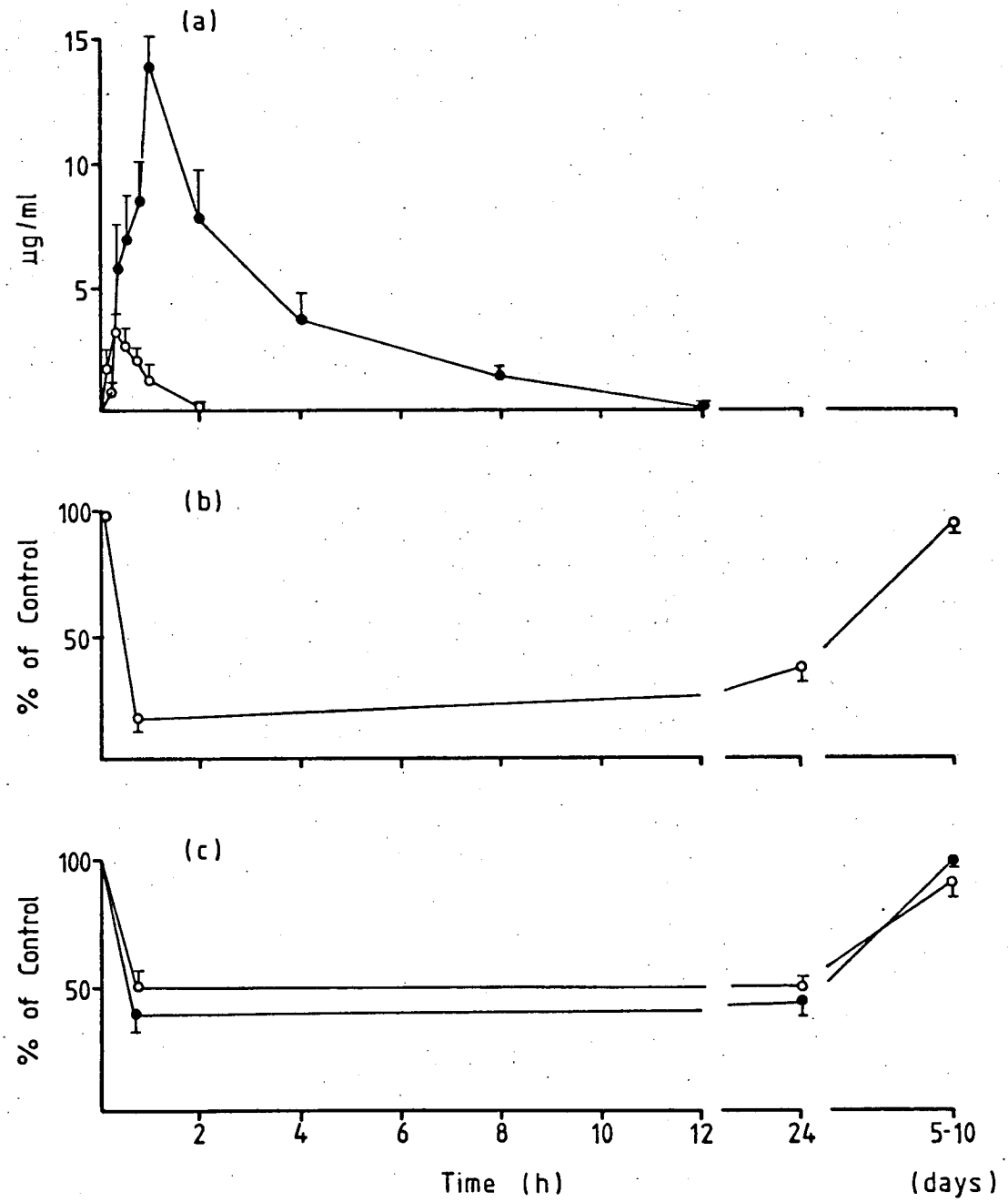


Figure 5.62 Plasma salicylate concentrations (a), extent of inhibition of MDA synthesis (b), and extent of inhibition of platelet aggregation (c) following ingestion of a single 300 mg dose of soluble ASA by human volunteers.
(a) plasma ASA concentrations, (○); plasma SA concentrations, (●).
(b) inhibition of MDA synthesis (○).
(c) inhibition of platelet aggregation induced by adrenalin (○) and collagen (●). Each point represents the mean ± se of the results for 5-9 volunteers.

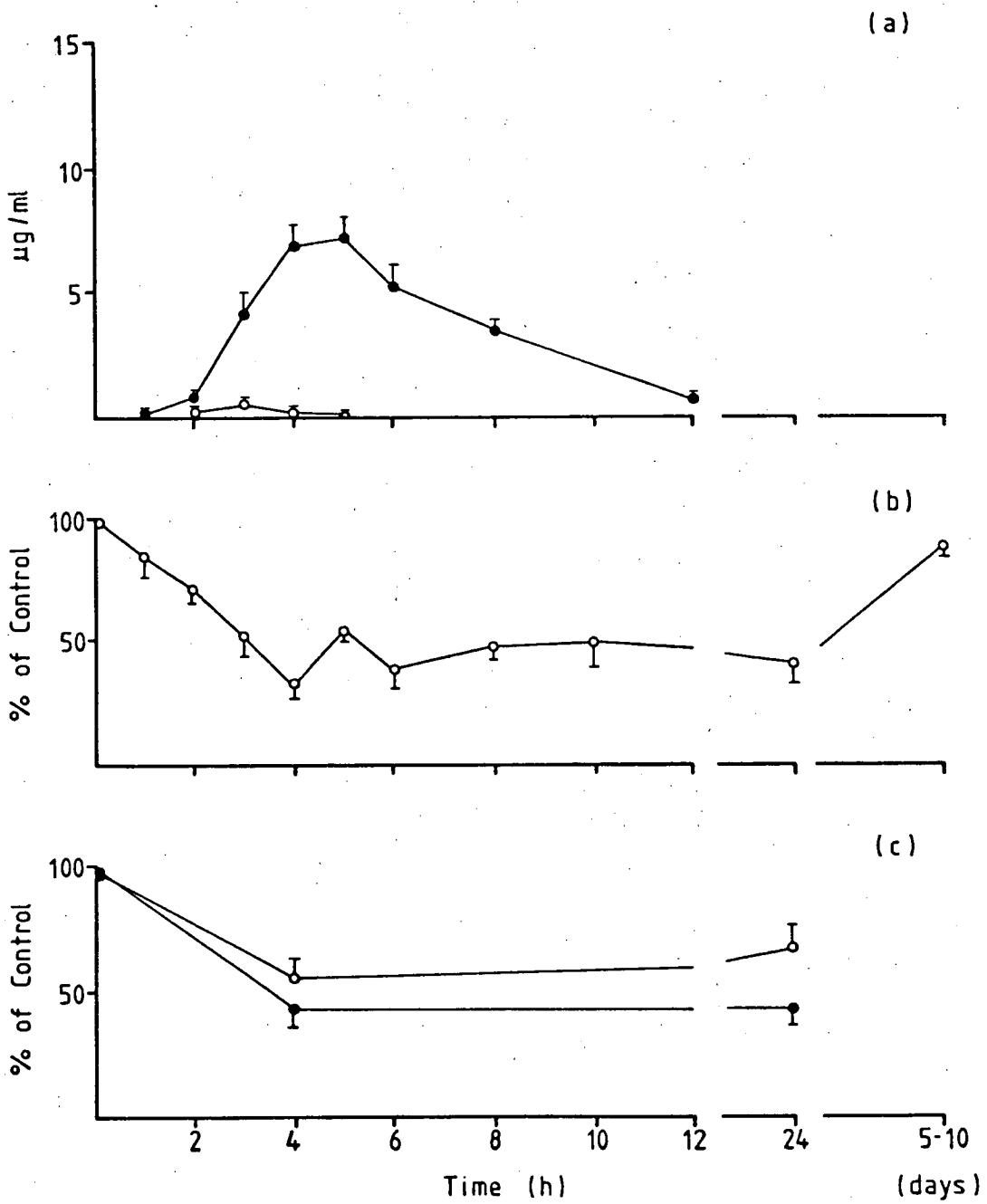


Figure 5.63 PLasma salicylate concentrations (a), extent of inhibition of MDA synthesis (b), and extent of inhibition of platelet aggregation (c) following ingestion of a single 300 mg dose of slow-release ASA by human volunteers.
(a) plasma ASA concentrations (○); plasma SA concentrations (●).
(b) inhibition of MDA synthesis (○).
(c) inhibition of platelet aggregation induced by adrenalin (○) and collagen (●). Each point represents the mean ± se of the results for 5-9 volunteers.

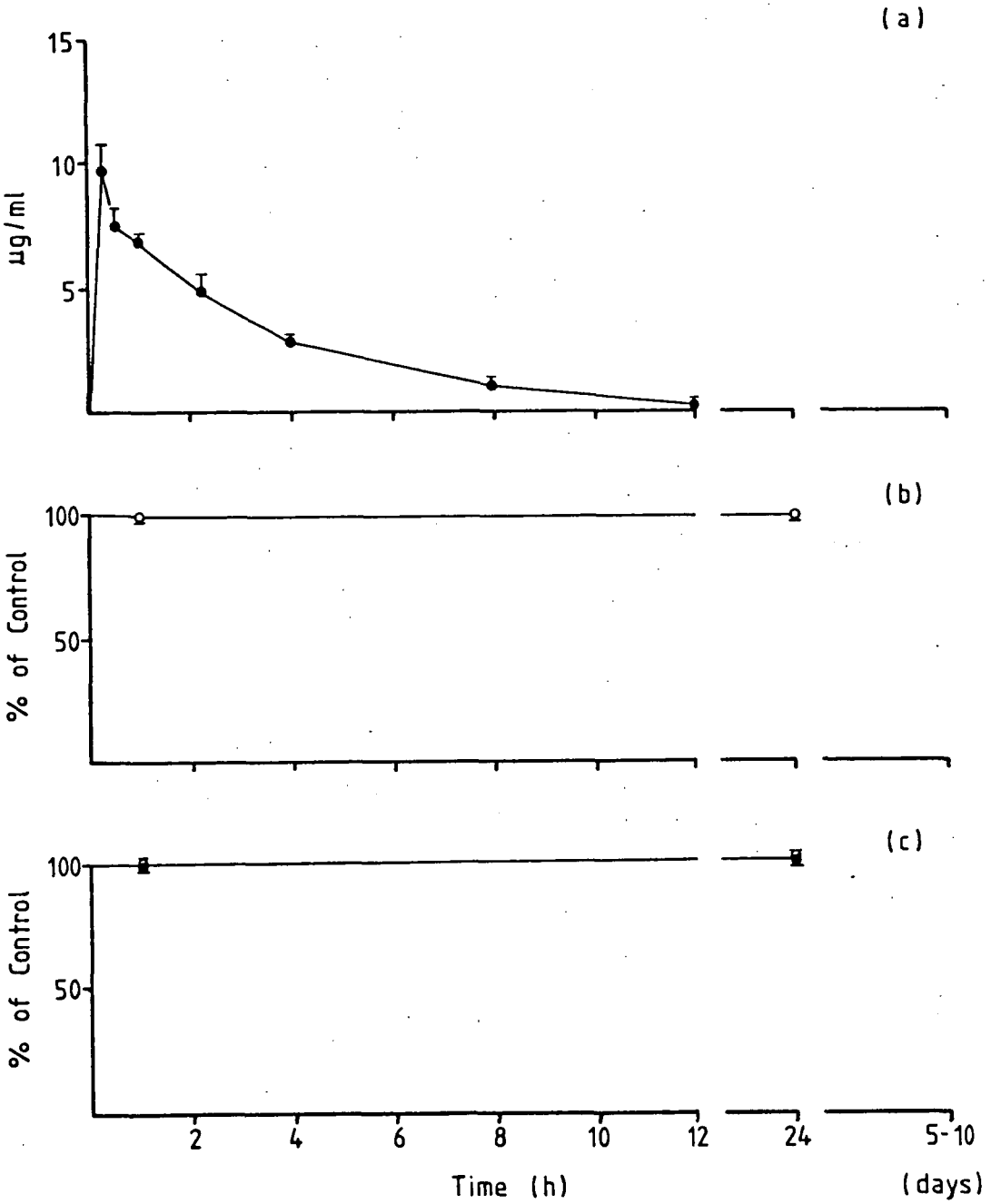


Figure 5.64 Plasma salicylate concentrations (a), extent of inhibition of MDA synthesis (b), and extent of inhibition of platelet aggregation (c) following ingestion of a single dose of salicylate (NaSA) equivalent on a molar basis to 300 mg ASA.
(a) plasma SA concentrations (●).
(b) inhibition of MDA synthesis (○).
(c) inhibition of platelet aggregation induced by adrenalin (○) and collagen (●). Each point represents the mean ± se of the results for 5-9 volunteers.

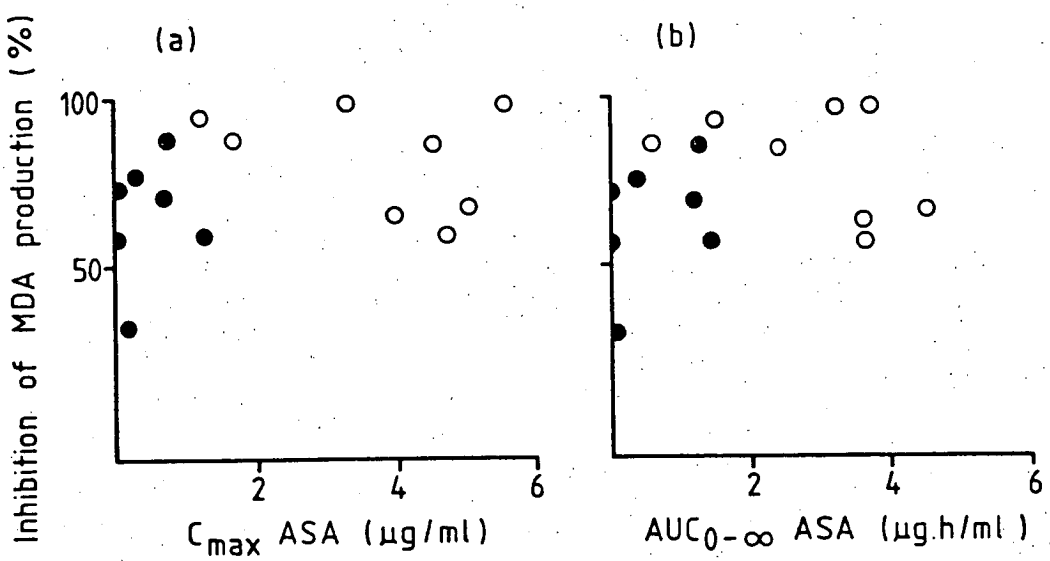


Figure 5.65 Peak ASA plasma concentrations (C_{max}) (a) and plasma AUCs (b) associated with the inhibition of MDA production after ingestion of a single 300 mg dose of soluble ASA (open symbols) or slow-release ASA (closed symbols) by human volunteers.

5.13 μg.hr/ml) dose forms (Figures 5.62 (a) and 5.63 (a)). A similar area (32.3 ± 2.9 μg.hr/ml) under the plasma SA concentration-time curve was found after ingestion of the sodium salicylate mixture (Figure 5.64 (a)).

Platelet Aggregation and MDA Production

The maximum inhibition by ASA of platelet aggregation induced by collagen and adrenalin was 40% and 70% respectively. Figures 5.62 (b) and 5.63 (b) show mean inhibition of MDA production, and Figure 5.62 (c) and 5.63 (c) show platelet aggregability after soluble and slow-release ASA respectively. The inhibition of platelet aggregability and MDA production was not significantly different for each of the formulations. Both forms of ASA produced a prolonged inhibition of platelet function. Full recovery of platelet function occurred at 5-10 days in all subjects (Figures 5.62 (b), (c); 5.63 (b)). An equimolar dose of sodium salicylate had no inhibitory effect either on MDA production or on platelet aggregation (Figures 5.64 (b), (c)).

Figure 5.65 shows plots for the inhibition of MDA production in individual subjects and the corresponding peak ASA concentrations and ACUs. It is apparent that the peak ASA concentrations and AUCs vary considerably between subjects for both the soluble and slow-release formulations, and, in fact, no relationships are apparent.

5.8.2. Continuous Dose Study

Plasma ASA and SA Concentrations

Plasma ASA concentrations were not detectable in any blood samples taken immediately before the last of any of the ASA doses

(trough sample) (Tables A46 to A51). Corresponding plasma (trough) SA concentrations were only detectable in one subject taking 650 mg ASA daily and in four subjects taking 1300 mg ASA, the maximum SA concentration being 0.4 μg/ml. Table 5.24 shows the mean plasma ASA and SA concentrations in samples taken 3 hr after (peak) the last ASA dose. ASA was not detectable in any subject after the ingestion of the 20 mg dose and in only two subjects given the 50 mg dose. Only one subject (taking 20 mg ASA) had undetectable levels of plasma SA. The detectable plasma concentrations of ASA appeared to be directly related to the dose of the slow-release ASA formulation for doses between 100 and 1300 mg (Table 5.24).

Platelet Aggregation and MDA Production

Figure 5.66 shows the log ASA dose-response curves for platelet aggregation in samples taken 3 hr after ingestion of the final slow-release ASA dose. With collagen as the inducing agent the maximal inhibition of platelet aggregation by ASA was 40%. With adrenalin as the inducing agent, ASA inhibited only the second phase of aggregation. The maximum inhibition of AA-induced aggregation (of 100%) was achieved with all the doses of ASA used. However, to achieve a maximal inhibition of collagen or adrenalin-induced aggregation, doses of about 100-200 mg ASA were required.

Figure 5.67 shows the log ASA dose-response curves for AA and NEM induced MDA production by platelets in samples taken 3 hr after ingestion of the final slow-release ASA dose. AA induced MDA production appeared to be maximally inhibited by 50 mg and above of the slow-release ASA product. Doses of about 200 mg and above of the slow-release product were required for maximal inhibition of NEM induced MDA production. The extent of inhibition of MDA production and

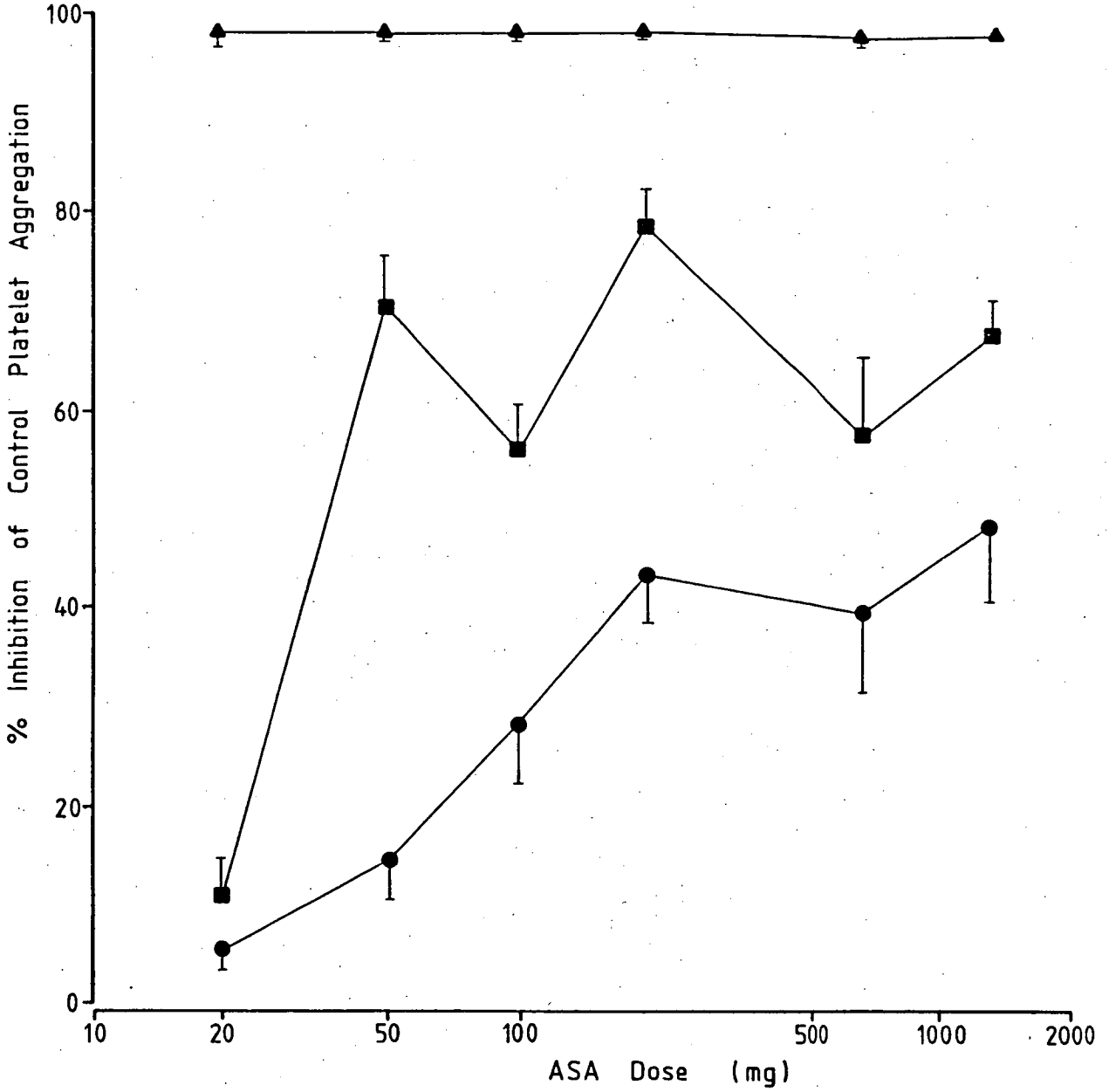


Figure 5.66 Dose-response curves for the effects of a slow-release ASA formulation on platelet aggregation induced by adrenalin (■), collagen (●) or arachidonic acid (▲) after daily doses of ASA for 7 days. Each point represents the mean ± se of the results for 3-8 volunteers.

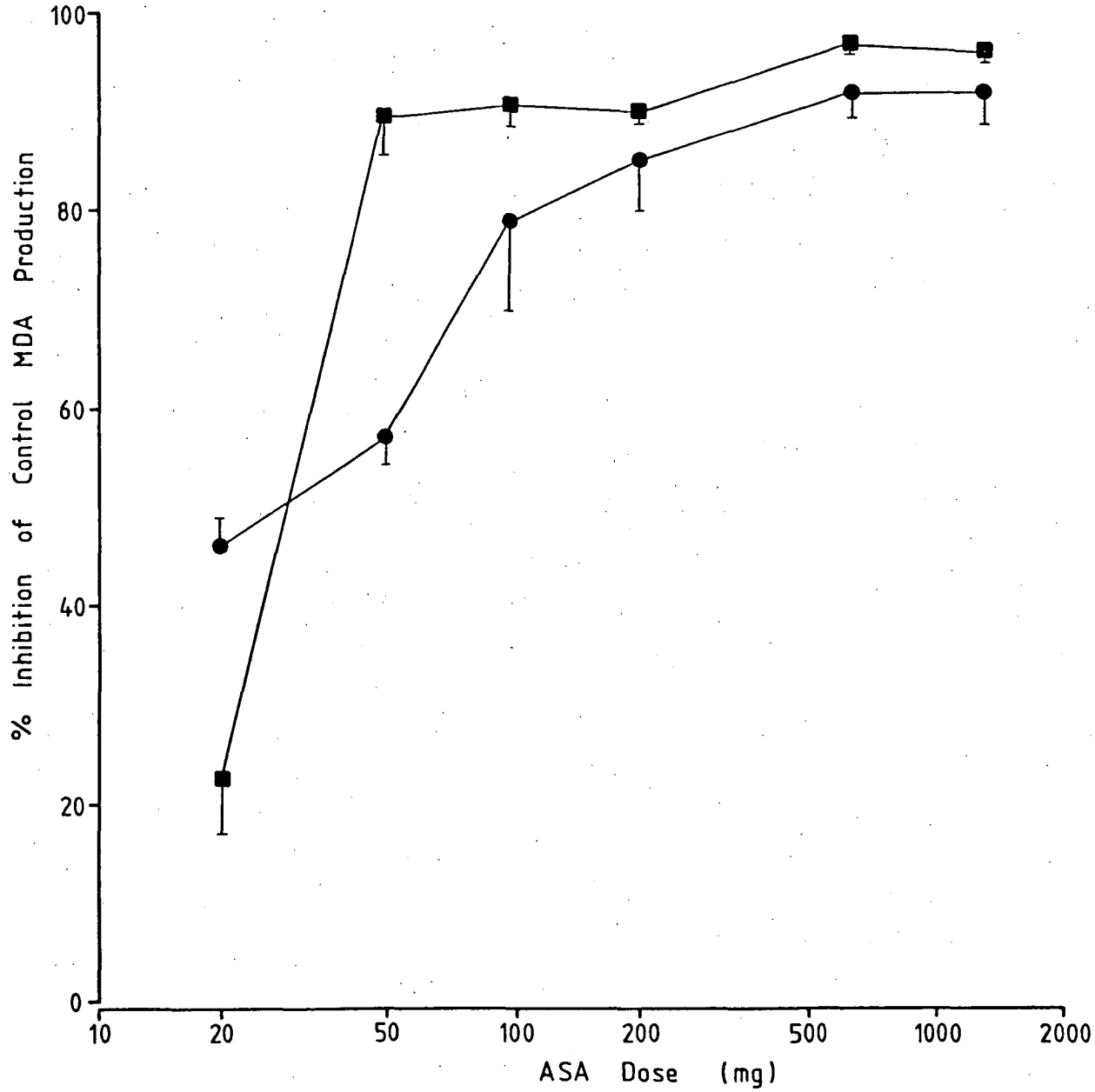


Figure 5.67 Dose-response curves for the effects of a slow-release ASA formulation on platelet MDA production induced by N-ethylmaleimide (●) or arachidonic acid (■) after daily doses of ASA for 7 days. Each point represents the mean ± se of the results for 3-8 volunteers.

Table 5.24 Plasma concentrations of aspirin (ASA) and salicylate (SA) 3 hr after ingestion of the seventh daily dose of Astrix (Mean ± SE, n=8).

Dose (mg)	ASA Concentration (µg/ml)	SA Concentration (µg/ml)
20	< 0.1	0.37 ± 0.09
50	< 0.1	0.56 ± 0.21
100	0.15 ± 0.07	1.06 ± 0.45
200	0.21 ± 0.06	2.37 ± 0.86
650	0.68 ± 0.26	4.00 ± 1.28
1300	1.51 ± 0.50	17.56 ± 4.81

Table 5.25 Platelet function in blood samples taken immediately before the last dose (trough sample, T) and 3 hr after the last dose (peak sample, P) in subjects ingesting a slow release ASA formulation (Astrix) daily for one week.

Dose (mg)	% Inhibition of control (Mean ± SE)									
	Adrenalin induced aggregation		Collagen induced aggregation		MDA synthesis	
	T	P	T	P	T	P
20	10.3 ± 3.6	11.2 ± 3.8	6.7 ± 2.5	5.6 ± 1.9	41.5 ± 4.8	23.0 ± 6.0
50	63.5 ± 8.9	70.2 ± 5.2	14.9 ± 5.0	14.7 ± 4.1	51.4 ± 8.4	89.6 ± 4.4
100	49.3 ± 10.6	54.7 ± 5.4	24.4 ± 8.6	28.4 ± 6.3	78.4 ± 3.2	91.1 ± 3.2
200	77.2 ± 3.4	78.0 ± 3.7	37.6 ± 3.7	43.4 ± 5.1	83.4 ± 5.5	89.4 ± 1.2
650	53.2 ± 4.8	56.7 ± 8.0	29.0 ± 7.9	39.2 ± 8.1	86.1 ± 1.7	97.2 ± 0.8
1300	65.7 ± 4.4	66.7 ± 3.4	37.5 ± 7.1	47.9 ± 7.8	87.8 ± 2.0	95.0 ± 1.7

Table 5.26 Platelet function in males (M) and females (F) for the blood samples taken 3 hr after the final daily dose of a slow release ASA formulation (Astrix) taken for one week.

Dose (mg)	% Inhibition of control (Mean ± SE)											
	Adrenalin induced aggregation				Collagen induced aggregation				MDA synthesis			
	M		F		M		F		M		F	
20	11.0 ± 1.0		6.5 ± 3.7		3.2 ± 2.2		8.0 ± 3.0		28.3 ± 12.3		16.0 ± 9.9	
50	74.5 ± 6.3		61.5 ± 7.5		9.7 ± 4.3		21.3 ± 6.3		86.0 ± 6.7		94.7 ± 4.8	
100	43.0 ± 24.8		63.5 ± 1.5		33.5 ± 12.2		23.3 ± 4.0		86.0 ± 4.9		96.2 ± 2.5	
200	76.7 ± 4.0		79.3 ± 6.9		41.0 ± 7.2		45.2 ± 7.9		84.5 ± 3.7		91.0 ± 0.91	
650	58.0 ± 9.0		52.5 ± 14.6		46.0 ± 12.4		32.5 ± 10.8		97.0 ± 0.8		97.5 ± 1.5	
1300	62.0 ± 7.0		69.5 ± 6.6		50.5 ± 9.9		45.2 ± 13.2		97.0 ± 1.4		92.0 ± 4.0	

platelet aggregation was similar in blood samples taken immediately before the last dose and 3 hr after the last dose (Table 5.25). No significant differences in platelet function between male and females were found for any of the slow-release doses used (Table 5.26).

CHAPTER 6

DISCUSSION

6.1. ANALYTICAL TECHNIQUES

6.1.1. Organic Nitrates

Specificity

Quantitation of GTN and its metabolites in biological fluids was found to be difficult. A high performance liquid chromatographic procedure based on the chromatographic conditions described by Crouthamel and Dorsch (1979) was suitable for high (supra-therapeutic) concentrations of GTN and its metabolites. Whilst gas chromatography assays of GTN are particularly sensitive for that compound (Yap et al., 1978; McNiff et al., 1981), the response of the electron capture detector to the GDNs is only about one half the response to GTN (Sokoloski et al., 1983). Moreover, a satisfactory method for resolving the GDNs on GC packings was not available when the current work was begun. The major problem in quantifying the di- and mononitrates by GC- electron capture is to obtain high and selective recoveries. The gas chromatograms using the hexane procedure of Yap et al. (1978) or the method used in this work (4.2.4.) display few interfering peaks to either GDNs or to GTN. However, only about 20% of the dinitrates are extracted into hexane using this procedure (Yap et al., 1978). Noonan and Benet (1982) and Needleman et al. (1972)

used the low extraction of the GDNs and the GMNs into petroleum ether, which is, in polarity, similar to hexane, as the method of quantifying labelled GTN. Ethyl acetate and diethyl ether also extract GTN and its metabolites from plasma (Needleman and Hunter, 1965; Crew and DiCarlo, 1968) but the gas chromatograms obtained by injecting concentrated extracts of ethyl acetate show a number of large interfering peaks (Rosseel and Bogaert, 1973). It is unlikely that the GMNs, if extracted from plasma by hexane, could be quantitated by such methods because of interfering peaks. It is even more unlikely if ethyl acetate were used because of the serious problem of interfering peaks extracted from plasma by that organic solvent (Rosseel and Bogaert, 1973). An attempt was also made to quantify GTN and GDNs simultaneously by GC-MS using negative ion chemical ionization methodology (Bignall et al., 1981). This method offered specific detection potential due to the unique $-ONO_2$ M/Z 62 ion. Although a great deal of time and effort was spent in trying to obtain a reproducible and reliable assay with this system, our attempts proved to be futile. The difficulty was due to variable and extensive adsorption of GTN to the column packing at the low carrier gas flow rates used for GC-MS. More recent work appears to have overcome these difficulties (Gerardin et al., 1982). Another problem was not having continuous access to a GC-MS and this method therefore was eventually assessed as being unsuitable for the studies reported in this thesis. The thermal energy analyzer has also been employed for organic nitrate analysis (Spanggord and Keck, 1980). It proved unsuitable due to cost considerations.

Sensitivity

The high performance liquid chromatographic procedure developed in this work (4.2.2) enables the separation of all organic nitrates studied with excellent recovery. Unfortunately, this analytical procedure proved to be unsatisfactory for the analysis of plasma samples containing small GTN concentrations. Concentrations of GTN in plasma ranged from 0.2 to 10 ng/ml in samples collected from subjects given therapeutic doses of GTN (Armstrong et al., 1980a; McNiff et al., 1981), i.e., using 0.2 ml plasma, a minimum detectability of about 40 pg GTN was required. Using a fixed wavelength detector at 214 nm, the limit of detectability was about 10 ng GTN on column.

Adequate specificity and sensitivity was achieved only by analyzing radiolabelled (^{3}H) GTN after separation by HPLC. Earlier work on GTN metabolism used thin layer chromatography (TLC) with or without a ^{14}C- labelled GTN (Needleman and Hunter, 1965; Lee, 1973). In those studies the GTN concentrations were very high (µg/ml) and greatly exceeded the usual therapeutic levels reported for GTN (Armstrong et al., 1980a; McNiff et al., 1981). The use of TLC to separate pg amounts of GTN for quantitation is likely to be unsatisfactory due to the volatility of GTN (Urbanski, 1965). In the current work, the recoveries of pg amounts of GTN and its metabolites from a C_{18} HPLC column were found to be incomplete and variable. The recovery for ^{3}H-GTN and its metabolites was markedly improved by adding large ng amounts of each unlabelled compound in samples to be injected.

Plasma GTN Concentrations

The GC assay of plasma GTN concentrations used in the current work was a modification of the method of Yap et al. (1978). As the recovery of GTN reported by Yap et al. (1978) could not be reproduced (5.1.1.), a simpler but less sensitive method was adopted (4.2.4.). The adopted technique provided the same recovery of GTN as reported by Yap et al. (1978) (i.e., 90%) and a similar precision (5.1.1.). Yap et al. (1978) reported a lower limit of detectability of 0.1 ng GTN/ml plasma using an injection technique for extracting GTN from plasma. This limit is about half that obtained with the present extraction procedure (5.1.1.). The present extraction procedure uses a more vigorous agitation technique. Injection of the resultant hexane concentrate gives a GC chromatogram not quite as 'clean' as that reported by Yap et al. (1978).

6.1.2. Salicylates

The HPLC assay of salicylates used in the current work (and originally developed in this laboratory) showed similar precision and reproducibility to that reported by Rumble et al. (1981).

6.1.3. Tests of Platelet Function

Platelet Aggregation

Variability of aggregation tests. The variability in aggregation results (5.1.3.) may be caused by variations in the endogenous concentrations of the inducing agents in samples collected from patients. It is recognized that tests of platelet aggregation using AA and

collagen as inducers are generally subject to less variability than those in which adrenalin is used. A large day-to-day variation in adrenalin induced response can occur in the same subject or in the same group of subjects (Table A2).

The large scatter of results observed for adrenalin induced aggregation is consistent with a reported coefficient of variation of 52% in one subject tested monthly for 6 months (Siess et al., 1981). Reasons for the day-to-day variability in adrenalin-induced aggregation are poorly defined. Stress is believed to be one determinant of the variability . One study (Arkel et al., 1976) showed that about half of the subjects delivering addresses to a conference had greatly diminished second phase responses to adrenalin-induced platelet aggregation immediately after delivering the address but 7-11 days later most had normal adrenalin-induced responses. In the present study the stress and trauma associated with blood sampling may also have contributed to the variable responses observed for adrenalin.

The published coefficients of variation of AA and collagen-induced platelet aggregation, reported by Siess et al. (1981) to be less than 15%, are in agreement with the intra- and inter-subject coefficients of variation found in the current study (Table A2).

Platelet MDA Production

Although the production of MDA by stimulating platelets with NEM has been widely used to assess platelet cyclooxygenase activity, NEM is a platelet stimulus of unclear physiological significance. Therefore, platelets were also stimulated with AA, the substrate for cyclooxygenase. The observed coefficients of variation for both inducers are in agreement with those of Stuart et al. (1975).

6.1.4. Plasma Protein Binding

As GTN and ASA are both susceptible to *in vitro* degradation by plasma enzymes (Maier et al., 1979; Rowland and Riegelman, 1967), it is essential to use a rapid plasma protein binding technique. Of the two commonly used techniques, equilibrium dialysis and ultrafiltration, ultrafiltration is quicker and hence this latter method was employed in this work.

Sorption of ASA and GTN by components of the ultrafiltration apparatus was evaluated as we had previously reported the high affinity of GTN for some plastics (Cossum et al., 1978; Roberts et al., 1980). Sorption of drugs by the filtration membrane for cellophane and other types of ultrafilters was reported by Moran and Walker (1968) and Spector et al. (1972). More recently Whitlam and Brown (1981) evaluated the ultrafiltration technique (using pressure, not centrifugation) for SA and other drugs and showed that SA was not bound by the membrane filter during control experiments using SA in buffer. In the current work SA, ASA, GTN, 1,3-GDN and 1,2-GDN were not sorbed by the ultrafiltration membranes (Amicon, YMT) tested or by the plastic apparatus.

6.2. DISPOSITION OF NITROGLYCERIN IN BLOOD

6.2.1. Metabolism by Erythrocytes

The present results confirm the results of Lee (1973) who reported that GTN was enzymatically degraded in erythrocytes to form the GDN metabolites. Noonan and Benet (1982) have also shown that the GDNs and GMNs are produced upon incubation of GTN and resuspended

erythrocytes in blood. All of these reports are at variance with results presented by Wu et al. (1981) and Sokoloski et al. (1983) who could not detect GDN metabolites following the loss of GTN from solutions incubated with resuspended erythrocytes and blood. It is not clear why those workers could not detect the GDNs even though they used an assay reportedly able to detect them. They concluded that GTN is degraded by some unknown physical process when in contact with blood components.

Despite those latter studies* current evidence suggests that the denitration of GTN in erythrocytes is enzymatic in nature. Evidence for a novel mechanism of GTN denitration comes from the recent work of Bennett et al. (1984). This interesting study showed that GTN denitration to GDNs in erythrocytes is due, at least in part, to the interaction of GTN with haemoglobin. When GTN was incubated with haemoglobin (ferrous), GDNs were detected, but pretreatment of the haemoglobin with carbon monoxide almost completely inhibited the metabolism of GTN to the GDNs. In incubations of GTN with erythrocyte lysate supernatant, pretreatment with carbon monoxide only partially inhibited GTN denitration. It is likely, therefore, that at least one mechanism other than interaction with haemoglobin is involved in the overall elimination of GTN in erythrocytes. Although there are at least two species of glutathione transferase present in erythrocytes (Marcus et al., 1978; Awasthi and Singh, 1984) one has been shown to have little, if any, activity towards GTN and the other has not been

* More recent work with an improved assay technique has shown the presence of the GDNs resulting from incubation of erythrocytes and GTN (T. Sokoloski, personal communication).

tested for activity towards GTN. Therefore, it is not presently known what other mechanism is responsible for GTN metabolism in erythrocytes.

GDNs were also lost from solutions incubated with resuspended erythrocytes. Initial studies using large concentrations of unlabelled GDNs showed that the larger GDN concentrations were lost at a slower rate than the smaller concentrations. These results are consistent with the report of Sokoloski et al. (1983) who showed that the half-lives of 1,3-GDN at concentrations of 10, 60, 180 and 480 ng/ml were 33, 40, 141 and 282 min, respectively.

The GMN metabolites of the unlabelled GDNs were not detected in the current study because of interfering peaks on the chromatograms. When the labelled GDNs were used in metabolic studies the GMNs were detected. This is best illustrated in Figure 5.13 which shows the generation of ^{3}H-GMNs. Noonan and Benet (1982) also showed that ^{3}H-GTN is metabolized by erythrocytes with the ultimate formation of the ^{3}H-GMNs. Unfortunately, Bennett et al. (1984) did not assay for GMNs during incubations of haemoglobin and GTN. Although the denitration of GDNs to form the GMNs is likely to occur in such incubations, this step awaits verification.

6.2.2. Metabolism by Plasma

The metabolism of GTN in human and sheep plasma occurs at a much slower rate than in erythrocytes. The half-lives of GTN at initial plasma concentrations of 10 and 50 ng/ml were about 50 min while half-lives of GTN at initial concentrations of 10 and 50 ng/ml blood or erythrocyte suspension were about 3.6 and 6 min, respectively. This

difference in the rate of GTN metabolism between plasma and erythrocytes had also been observed by Armstrong et al. (1980c), Noonan and Benet (1982), and Sokoloski et al. (1983).

6.2.3. Concentration Dependent Nature of Metabolism

It is clear from Figure 5.14 and Tables 5.7 and 5.8 that the rate of metabolism of GTN by erythrocytes is concentration dependent, the large GTN concentrations being metabolized at a slower rate than the smaller GTN concentrations. Similar findings have been reported by Noonan and Benet (1982) and Sokoloski et al. (1983). The smallest GTN concentration studied by those groups was 17 ng/ml and 10 ng/ml, respectively, with the half-lives of disappearance of GTN being 15 min and 4 min, respectively. In the current work initial GTN concentrations of 2 and 0.8 ng/ml suspension or blood, which are near the lower end of the therapeutic range in man, were found to be metabolized with half-lives being about 3 min. An explanation of the concentration dependent nature of the *in vitro* rate of metabolism of GTN in blood is not readily forthcoming. If Michaelis-Menten kinetics were operative then the slope of the plots of % GTN remaining versus should have increased with time. Even for extended incubation times when the limits of GTN detectability were reached the plots remained linear (results not shown). This unusual kinetic behaviour could be a result of the use of intact cells. When homogenates of other tissues were used no such concentration dependent metabolism of GTN was observed. This could suggest that the endogenous co-factors of the erythrocyte were depleted at different rates for larger GTN concentrations. Finally, Bennett et al. (1984) suggest that at least two mechanisms are responsible for denitration of GTN in erythrocytes. The unusual

kinetics of GTN denitration observed in erythrocytes may be a result of simultaneous denitration processes.

6.2.4. Concentration Independent Nature of Distribution

The distribution of a drug within blood is a function of plasma protein binding, partitioning into erythrocytes and the haematocrit (Rowland and Tozer, 1980). The initial study in the current work (Figure 5.14) showed that although the rate of GTN metabolism was concentration dependent, the rapid initial uptake of GTN by erythrocytes was relatively constant (a drop in GTN concentration of about 20-30%). In the study designed to accurately quantitate that initial drop the haematocrit was set at 0.45 and the plasma protein binding of GTN and the affinity of GTN for the erythrocytes were found to be constant over a wide GTN concentration range. Thus it would be expected that the blood/plasma (B/P) concentration ratio of GTN would be constant over a wide GTN concentration in any one subject. Noonan and Benet (1982) found that the B/P ratio of GTN was about 0.65 for an initial GTN concentration of 136 ng/ml blood and throughout the time course of degradation. The B/P ratio can be calculated using the equation (Rowland and Tozer, 1980);

$$\frac{C_b}{c} = 1 + H\,(F_u.a - 1) \qquad (6.1)$$

where C_b = blood concentration of the drug,

c = plasma concentration of the drug,

H = haematocrit,

f_u = fraction of drug unbound in plasma and

a = affinity of the erythrocyte for the drug.

Calculations using the erythrocyte/plasma partition coefficient as an index of the erythrocyte affinity for GTN give a B/P ratio of 0.91. Experimental data (Figure 5.18) give a B/P ratio of 0.75 (calculated from the dpm found in the plasma when separated from the erythrocytes). These results are consistent with those of Noonan and Benet (1982) and show that GTN is not concentrated in the erythrocyte. Furthermore, the B/P ratio for GTN metabolites of 0.9 - 1.2 reported by the above workers is consistent with reduced plasma protein binding of 1,2-GDN and 1,3-GDN when compared to GTN (Tables 5.7 and 5.8).

6.2.5. Pharmacokinetic Implications of Nitroglycerin Denitration In Blood

The limited information available on the pharmacokinetics of GTN in man suggest that the contribution of GTN clearance by blood to the overall body clearance is probably small. McNiff et al. (1981) using an estimated half-life of metabolism of 3 min for GTN in blood concluded that this route of clearance could only account for < 3% of the overall body clearance. Thus, GTN metabolism by blood is probably of major importance in the estimation of plasma GTN concentrations as significant amounts of GTN may be degraded prior to analysis. This will be particularly important in bioavailability studies of GTN from ointment and transdermal delivery systems when plasma GTN concentrations will be invariably less than 1 ng/ml (Black, 1982). Figure 5.18 shows that if an enzyme inhibitor is not added to the blood immediately on sampling from a patient and if there is delay in centrifuging the blood, degradation of GTN will occur despite the blood being placed in a container precooled to 2°C. A delay of only 3 min between sampling and centrifuging could result in a loss of 30% of the

GTN present if no iodoacetamide (or other effective agent) is added to the blood immediately on sampling.

A recent study by Rey et al. (1983) highlights the ramifications of not taking sufficient care to ensure the in vitro stability of GTN. The method employed by those workers involved blood sampling, after administration of GTN, and storing the blood samples for 10 min at 4°C before centrifugation to obtain plasma for GTN analysis. No mention was made of using an enzyme inhibitor to prevent the in vitro degradation of GTN in blood. Figure 5.18 shows that the metabolism of GTN (initially 1.5 ng/ml blood) by blood proceeds at 2°C, albeit slowly, but at a sufficient rate to result in an underestimation of GTN plasma concentrations by about 25% after 10 min storage. Rey et al. (1983) comment that the GTN plasma concentrations obtained from their study were lower than expected based on the results of Armstrong et al. (1979) for similar GTN doses. Armstrong et al. (1979), although not using an enzyme inhibitor, immediately centrifuged blood samples to obtain plasma for GTN analysis.

GTN is also degraded in plasma. Silver nitrate is used extensively to reduce the degradation of GTN by plasma during work-up for assay or during storage at -20°C (Yap et al., 1978; Maier et al., 1979); however, it is not used to prevent GTN degradation by blood because it causes gelling of the blood sample. The efficiency of iodoacetamide in inhibiting degradation of GTN by plasma on storage at -20°C has not been evaluated although Taylor et al. (1981) found that iodoacetamide (final concentration of 4 mM) prevented the degradation of GTN in plasma for up to 5 hr at room temperature. They also reported that iodoacetamide did not interfere with the determination

of plasma concentrations of GTN using the assay of Yap et al. (1978). This aspect was confirmed for sheep plasma in the current work.

GTN is not the only drug to undergo *in vitro* metabolism in blood. Rowland and Riegelman (1967) showed that aspirin is hydrolyzed to salicylic acid *in vitro*. More recently, Chen et al. (1983) have shown that procainamide is metabolized to N-acetylprocainamide when stored in blood at 5°C and 25°C. It appears then that drug stability needs to be ascertained not only in formulations intended for administration to patients but also in blood and plasma samples taken from those patients in order to ensure proper dosage regimen adjustments based on correct pharmacokinetic calculations.

6.3. PHARMACOKINETICS OF NITROGLYCERIN IN SHEEP

6.3.1. Continuous and Bolus IV Doses

Delivery System

During IV infusions, steady state plasma drug concentrations (C_{ss}) are dependent upon the rate of administration of the drug and the clearance of the drug (Rowland and Tozer, 1980). The rate of administration is usually well defined. However, if the drug being infused interacts with the infusion system, the actual rate of administration is less than anticipated. GTN interacts with the plastics of standard delivery systems made of polyvinyl chloride and cellulose propionate (Cossum et al., 1978; Roberts et al., 1980; Baaske et al., 1980). However, GTN has little affinity for polyolefins such as polyethylene (Cossum et al, 1978; Baaske et al., 1982) and so these plastics must be used for GTN infusions to prevent

the loss of the drug during infusions. Other drugs known to interact with the plastics of conventional administration sets are isosorbide dinitrate (Cossum and Roberts, 1981), diazepam (Parker and MacCara, 1980) and chlormethiazole (Tsuei et al., 1980). These drugs are, however, compatible with polyolefin systems (Cossum and Roberts, 1981).

Sampling

A further potential problem in using GTN in this work was an interaction of GTN and the plastic catheters used for blood sampling. Table 5.13 shows that losses due to such an interaction were minimal, probably because:

(i) the catheters were made of a polyolefin substance; and

(ii) the flow rate used in this validation was high (40 ml/min).

High flow rates were used to mimic the experimental situation where blood samples are withdrawn rapidly. Jacobi et al. (1983) reported a transient loss of GTN to central venous pressure catheters using a very slow flow rate of drug solution of 30 ml/hr. Table 5.13 also shows that when the flow rate of GTN drug solution being drawn through the plastic catheters was reduced to 5 ml/min (as would happen during partial blockage of blood sampling lines) the loss of GTN was still less than 2%. The GDNs and GMNs showed no losses during contact with plastic catheters.

Plasma Concentrations After Bolus Injections

GTN disappeared from arterial venous plasma in a bi-exponential manner after administration of bolus injections over 1 min. Needleman et al. (1972) found that the blood clearance of ^{14}C-GTN in rats was

bi-phasic following IV bolus doses. In contrast, no distribution phase was reported for GTN given intracardially (700 µg/kg) to rats (Yap and Fung, 1978). Idzu et al. (1982) and Miyazaki et al. (1982) both reported bi-phasic decay profiles of GTN venous plasma concentrations after bolus intravenous doses of GTN to humans and dogs, respectively.

The half-lives of the two phases of GTN plasma concentration decay were approximately 0.8 min and 2.0 min, respectively, for all but the largest bolus dose of GTN in arterial and venous plasma. The half-lives of the α and β phases of decay after administration of the largest bolus dose were about 1.0 and 3.0 min, respectively. These times are similar to 0.45 min and 4.25 min after a dose of 7 µg/kg in conscious dogs (Miyazaki et al., 1982), 0.37 min and 4.95 min after a dose of 4 µg/kg in anaesthetized humans (Idzu et al., 1982) and the β phase half-life of 4.2 min in rats that received an intra-cardial bolus dose of 700 µg/kg (Yap and Fung, 1978).

Plasma Concentrations After Infusions

Steady-state GTN venous and arterial plasma concentrations were generally achieved within 20 min after beginning an infusion. McNiff et al. (1981) reported that steady-state or near steady-state GTN concentrations in venous plasma were reached within 30 min after starting a GTN infusion at the rate of 0.23 µg/min/kg in 3 of 9 normal human subjects. The average venous C_{ss} found by McNiff et al. (1981) was about 0.4 ng/ml. The average C_{ss} of about 0.5 ng/ml in venous plasma of sheep receiving GTN infusions at the rate of 0.4 µg/min/kg (Figure 5.33) is of this order. Armstrong et al. (1982) found that GTN infusions at a rate of about 0.28 µg/min/kg resulted in average

venous C_{ss} of 0.90 ng/ml and average arterial C_{ss} of 2.6 ng/ml in four patients with congestive heart failure.

Considerable inter-individual differences in GTN plasma levels were observed in human patients or volunteers receiving continuous IV GTN infusions (Armstrong et al., 1980a; Imhof et al., 1982; McNiff et al., 1981; Wei and Reid, 1979). It has been pointed out by McNiff et al. (1981) that the amount of GTN in the plasma compartment probably accounts for about 1% of the total amount of GTN in the body. Minor fluctuations in the amount of GTN in the tissues may cause major fluctuations in the plasma GTN concentrations. In this respect, changes in posture may contribute to fluctuations in GTN plasma concentrations (Curry and Kwon, 1985). Sheep used in this work were anaesthetized and so completely immobilized. This factor may have contributed to the small variations observed in apparent steady-state GTN plasma concentrations of blood sampled from each of the six sampling sites in sheep. Moreover, stabilization of GTN in blood by iodoacetamide after sampling may also have contributed to the small inter-individual variations in apparent steady-state GTN concentrations.

The decay in plasma GTN concentrations after termination of GTN infusions in sheep was found to be monoexponential for a GTN dose of 0.4 μg/min/kg but biexponential for the two larger doses. The second phase of the GTN decay curve after infusions of 0.4μg/min/kg was not observed due to the lack of sensitivity of the GC assay used to analyze plasma GTN concentrations (0.2 ng/ml). McNiff et al. (1981) also reported a monoexponential decay of GTN after infusions in humans using a GC assay with a detection limit of 0.05 ng/ml. A half-life of about 3 min was reported by those workers. The half-lives for the two phases of GTN disappearance from venous plasma were about 0.75 min and

3-4 min, respectively. It could be inferred that the half-life calculated by NcNiff et al. (1981) comprises components of the distribution and elimination phases. Miyazaki et al. (1982) reported the half-lives of the α and β phases of decay in venous plasma GTN concentrations in anaesthetized dogs to be 0.50 and 4.95 min, respectively.

6.3.2. Arterial-Venous Plasma Nitroglycerin Gradients

A pronounced difference in GTN plasma concentrations exists at the different sampling sites of sheep given GTN by infusion. These results are consistent with the report of Armstrong et al. (1982) that a substantial gradient in GTN plasma concentrations existed between the radial artery and a peripheral vein in humans receiving continuous infusions of GTN. Calculations of systemic venous plasma clearance in the current work give values which greatly exceed normal cardiac output in sheep (about 2.5 l/min). Blood clearance is even greater than plasma clearance and is obtained by multiplying plasma clearance by 1/0.83 (0.83 is the average blood/plasma ratio of the two values calculated in this work, 0.75 and 0.91, Section 6.2.4.). Systemic arterial plasma clearance values were also greater than cardiac venous clearance. These results suggest that a substantial proportion of GTN is cleared by extra-hepatic routes.

Extraction Across the Leg

The results from the tissue homogenate experiments show that the observed arterial-venous GTN concentration gradient is likely to be a result of GTN metabolism in the leg tissues. Metabolism of GTN across the hind leg has not been reported previously although Runciman et al. (1982) have reported that lignocaine, pethidine and chlormethiazole

were all substantially extracted across the hind limb of sheep. Kreye and Reske (1981) reported an arterial-venous gradient for nitroprusside across the hind limb of the anaesthetized rat and suggested that this site was partially responsible for the rapid in vivo inactivation of nitroprusside.

The mean availability of GTN across the hind leg of the sheep was between 0.12 and 0.29 for increasing GTN doses delivered as continuous IV infusions, and 0.21 and 1.04 for GTN doses delivered as bolus injections. These values for availability were associated with decreased MRTs; however, the decrease in MRT at the largest GTN doses delivered by either continuous infusion or bolus injection did not reach statistical significance, owing at least in part to the variability of individual MRTs (Tables 5.14 and 5.16).

The contributions of haemodynamic (Section 6.4) and metabolic changes to the observed dose-dependent increases in GTN availability may not be easily separated. However, some separation may be gauged by assuming the disposition of a drug in an organ is described by a particular pharmacokinetic model. If the disposition of a drug is described by a well stirred model, availability (F) and mean residence times (MRT) can be related to the volume of distribution of the drug (V) in the organ, the organ blood flow (Q), the fraction of the drug unbound in blood (f_u) and the intrinsic clearance (Cl_{int}) by the organ using equations described by Weiss (1982):

$$F = \frac{Q}{Q + f_u \, Cl_{int}} \qquad (6.2)$$

and

$$MRT = \frac{V}{Q + f_u \, Cl_{int}} \qquad (6.3)$$

On re-arrangement of these two equations it is seen in equation 6.4 that the MRT of a solute in an organ is inversely proportional to the blood flow in that organ:

$$MRT = \frac{FV}{Q} \qquad (6.4)$$

The decreases in MRT of GTN at higher GTN doses corresponds to increases in cardiac output (and therefore hind leg blood flow) for GTN doses administered by continuous infusion. Increased blood flow in the leg does not, however, account completely for the observed decreases in MRT and associated increases in availability of GTN with larger GTN doses since larger GTN doses delivered by bolus injection reduced the cardiac output, and supposedly hind leg blood flow. In addition, the cardiac output never increased to any appreciable degree after baseline levels had been re-established following the fall in cardiac output induced by larger GTN bolus doses.

Results from the *in vitro* experiments showed that muscle tissue is capable of metabolizing GTN to the GDNs and GMNs. Moreover, the rate of GTN metabolism is reduced in the presence of GDNs *in vitro* and the clearance of GTN is reduced by the co-administration of GDNs *in vivo* (see below). These results suggest that it is most unlikely that only haemodynamic parameters influence the disposition of GTN *in vivo*. They further suggest that the well-stirred model does not completely describe the disposition of GTN.

Extraction Across the Lungs

The lungs were found to be a site of GTN clearance in the sheep. During continuous GTN infusions, the mean GTN availability across the lungs ranged from 0.53 to 0.72, indicating that substantial amounts of

GTN were being lost in that tissue. Armstrong et al. (1982) have shown that in humans receiving continuous infusions of GTN there was an extraction of about 16% of GTN during passage through the lungs. While the concentrations of GDNs in blood leaving the lungs were greater than in blood entering the lung for all times studied, this may have been due to a distribution phenomenon whereby the lung could "trap" GDNs produced at other body sites early during the infusion, releasing them back into the plasma at subsequent times.

In vitro experiments verified that lung tissue is capable of metabolizing GTN to GDNs and GMNs. Those results are consistent with results presented by Heinzow and Ziegler (1981) who showed that 10% of GTN was lost from solutions being perfused through isolated rat lungs with the concomitant formation of GDNs. Another organic nitrate drug, isosorbide dinitrate, has recently been shown to be metabolized by rabbit lung homogenates and in perfusion experiments (Mayer et al., 1983).

The variation in the availability of GTN across the lung with infusion rate may be related to changes in pulmonary blood flow brought about by reductions in the systemic venous return. Since pulmonary blood flow is, in part, determined by the systemic venous return, alterations in GTN availability at the highest GTN dose may reflect a reduction in pulmonary blood flow (as opposed to pulmonary artery flow or cardiac output).

Extraction Across the Liver

It has been known for many years that GTN is metabolized in the liver (Oberst and Snyder, 1948; Heppel and Hilmoe, 1950) to form the GDNs and GMNs (Needleman and Krantz, 1965). The results of the

current work are consistent with those reports. Based on those early reports and more recent work showing that the bioavailability of GTN given orally is very small due to extensive first-pass metabolism (Needleman et al., 1972; Needleman, 1973; Yap and Fung, 1978), GTN is not routinely administered in oral preparations.

The current results show substantial amounts of GTN in blood leaving the liver via the hepatic vein. This finding is contrary to what was expected based on the reports of Needleman and co-workers. Needleman (1973) wrote, "Orally administered organic nitrates are absorbed into the portal circulation and are rapidly and completely degraded by a liver enzyme before reaching the systemic circulation and therefore can have little chance of producing vasodilation." In an editorial, Krantz and Leake (1975) questioned Needleman's assertion that orally administered GTN is completely metabolized in the first-pass through the liver. They raised the point that if Needleman was correct then William Murrell's anginal patients would have failed to benefit by taking GTN orally, a route which was known to alleviate anginal pain. It may be that for larger GTN doses, the hepatic metabolism of GTN is reduced by co-factor depletion or by metabolite inhibition (see Section 6.3.4.). The concentration dependence of GTN clearance by the liver in vivo was not reflected by the in vitro findings. Table 5.11 shows that the metabolism of GTN by liver homogenates was independent of GTN concentration. The results imply that in vitro metabolism of GTN by sheep tissue homogenates was not limited by co-factor depletion.

Extraction of GTN by the liver increased as the dose of GTN increased so that at the larger GTN infusion rate the availability of GTN was about 0.5 when comparing its concentration in the portal and

hepatic veins. This availability calculation assumed no contribution by the hepatic artery. The estimate is likely to be larger in reality due to the 30% contribution of the hepatic artery flow to the blood flow in the hepatic vein (Zakim, 1981) and assuming that GTN delivered to the liver via arterial and venous bloods is subjected to the same metabolizing systems.

It is uncertain if the apparent concentration-dependent clearance of GTN by the liver could be explained by changes in liver blood flow induced by GTN. Firstly, Colley and Sivarajan (1984) showed that liver blood flow of dogs was unaffected by GTN infusions during halothane anaesthesia. Dumont et al. (1982), however, reported that GTN infused to reduce the blood pressure of conscious dogs by 20 mm Hg decreased the liver blood flow by about 20%, but those workers measured the blood flow of the hepatic artery only. Secondly, Blei et al. (1984) found that portal vein ligation, which reduced the liver blood flow by up to 40%, had no effect on the apparent systemic clearance of GTN in pentobarbitone-anaesthetized rats. It is not known what the effects of pentobarbitone anaesthesia, surgery and GTN infusions are on liver blood flow of sheep as such measurements were not made during the experiments. Therefore, even if there is a GTN dose-dependent increase in liver blood flow it is unlikely that it would explain completely the observed increase in availability of GTN with increasing GTN doses. A more likely explanation is that the metabolism of GTN is reduced at larger GTN doses by either depleting co-factor stores or by inhibition by the GDNs (see below). Fung et al. (1984a) have suggested that the presence of at least two enzyme systems each having different affinities and/or capacities for GTN may explain the dose-dependent increase in oral bioavailability of GTN in

the rat. Such a proposal may also help to explain the dose-dependent availability of GTN across the liver of sheep.

Extraction by Blood Vessels

Although no specific studies were performed to assess the relative uptake of GTN by arteries and veins, studies did show that sheep arteries and veins are capable of metabolizing GTN to the GDNs (Table 5.11). These results are in accord with the recent findings of Fung et al. (1984b) that rat blood vessel segments metabolize GTN to the GDNs, and ISDN to the isosorbide mononitrate metabolites. Those workers demonstrated a substantial "first-pass" uptake of GTN by blood vessels after administration of GTN into various vascular sites: the segments of blood vessel nearest the site of administration of GTN had larger GTN concentrations than segments further "downstream" of the site of administration. Their results also suggested a lower affinity of GTN for aorta but it was unclear whether this should be interpreted as meaning that arterial tissue is generally responsible for less GTN uptake than venous tissue.

The current results and those of Fung et al. (1984b) are in conflict with those of Armstrong et al. (1980b) who, despite using a sensitive assay, did not detect GDNs in the perfusate of isolated dog blood vessels. This may have been because the GDN metabolites distributed into the blood vessels upon formation, or alternatively the segments of blood vessels may have been too small to produce detectable amounts of GDNs.

Metabolism of GTN in an individual organ is dependent on the enzyme activity within the cells of the organ and in the blood vessels perfusing the organ. In some organs, e.g. capillary beds, blood

vessels are numerous and may play a major role in GTN metabolism. What the contribution of the blood vessels is to the overall metabolism of GTN remains to be investigated. An increase in the blood flow in a vessel would, however, be expected to be associated with a reduced extraction of GTN by that vessel. An analogy is the finding of a reduced extraction of GTN by polyvinyl chloride infusion tubing at faster GTN solution flow rates (Cossum et al., 1978).

The metabolism of GTN by blood vessels may also be of importance in the present hypotheses of the mechanisms by which GTN causes vasodilation. Several hypotheses have been proposed to explain the mechanism by which GTN and other organic nitrate drugs relax vascular smooth muscle (Bennett and Marks, 1984). According to the theory of Needleman and Johnson (1973) and Needleman et al. (1973), GTN reacts with sulphydryl groups of receptors in the vascular smooth muscle. The reaction converts the nitrate receptor to the disulphide form, at the same time reducing GTN to a denitrated (GDN) form with the release of a nitrite ion.

Ignarro et al. (1981) have suggested a scheme in which GTN is reduced to one of the GDNs with the release of a nitrite ion, which by a series of reactions forms a reactive intermediate. The formation of cGMP is enhanced by the reactive metabolite and it is this increased level of cGMP which causes relaxation. The results of the current work are consistent with both of these hypotheses since both hypotheses require the formation of the GDN metabolites. However, Fung et al. (1984b) point out that the rapid onset of action of GTN in intact animals requires that there be a similarly rapid denitration of GTN in the blood vessels. Those workers recommend further studies to examine

whether denitration of GTN completely accounts for its vasodilator action.

6.3.3. Arterial-Venous Glyceryl Di- and Mononitrate Gradients

Because of the difficulty in measuring plasma GDN concentrations there is a scarcity of literature on the topic of GDN pharmacokinetics. Most workers have used large doses of radio-labelled GTN (^{14}C-GTN) in experiments with rats, giving high blood GTN concentrations (μg/ml) (DiCarlo et al., 1968; Johnson et al., 1972). In those studies either the GDNs were measured as a single substance in plasma (Johnson et al., 1972) or as separate substances in urine and the results used to give an indirect measure for plasma (DiCarlo et al., 1968). In the latter study, both GDN isomers were present in all urine samples, the concentration of the 1,2-GDN always being greater than that of the 1,3-GDN.

By using HPLC and ^{3}H-GTN in the current work, it was possible to measure separate plasma GDN concentrations and combined GMN concentrations following the infusion of a GTN dose (0.4 μg/min/kg) commonly used clinically in humans. The bi-exponential decay profile for the 1,3-GDN and 1,2-GDN had a terminal half-life of about 40 and 35 min, respectively, which compare to the terminal half-lives in dogs of 48.5 and 40.6 min reported by Miyazaki et al. (1982). Those workers infused GTN into dogs at a rate of 6 μg/min/kg and measured plasma GDN concentrations by gas chromatography-mass spectrometry. They also found that plasma concentrations of 1,2-GDN were about 3- fold that of the 1,3-GDN, a result similar to that found in the current work. Estimates of the half-lives of the α phase of plasma GDN decay profiles varied with the site of sampling; however, the half-lives in

venous plasma were about 10 min and 15 min for the 1,2-GDN and 1,3-GDN, respectively. Corresponding values reported by Miyazaki et al. (1982) for venous plasma were 8.1 min and 8.5 min.

The analytical procedure used for this work did not fully resolve the GMNs and so they were measured as one peak. Moreover, the plasma GMN concentrations had not decreased at the end of blood sampling and so their pharmacokinetic parameters could not be estimated. Spanggord and Keck (1980) using a HPLC with thermal energy analyzer method for quantifying organic nitrates found that the predominant plasma GMN was 2-GMN in dogs given GTN orally. No estimate of the pharmacokinetic parameters of the GMNs could be made in that study because of the incomplete metabolism of the GDNs.

Metabolite Gradients Across the Leg

The arterial-venous gradient in plasma GDN concentrations was reversed after the termination of the GTN infusion. This resulted in plasma AUCs for the GDNs which were only slightly less in the venous plasma than in the arterial plasma. The calculated values for availability were about 0.9 for the GDNs (Table 5.19) indicating that they were metabolized in the leg. Confirmation that muscle could degrade GDNs to GMNs was obtained from *in vitro* experiments (Table 5.11). Thus, muscle appears to contain the necessary enzymes for the denitration of GTN to the GMNs.

An arterial-venous gradient exists for plasma GMN concentrations (Figure 5.47). This gradient was diminished during the GTN infusion so that by about 60 min after the GTN infusion ceased there existed a venous-arterial plasma GMN gradient. The earlier arterial-venous gradient most probably represents distribution of GMNs into the muscle

tissue while the later venous-arterial gradient represents distribution of GMNs back from the tissues into the plasma. Unfortunately, the experiments were not conducted over a long enough time period for quantitating the availability of GMNs across the leg. As the muscle probably lacks conjugating enzymes (Gram, 1980) it is most probable that the availability of GMNs would have been unity.

A very small portion of GTN is excreted in rat urine as the glucuronic acid conjugate of GMN (DiCarlo et al., 1968). It is possible that very small amounts of GMNs are metabolized also to glycerol since DiCarlo et al. (1968) measured ^{14}C- glycerol in the urine of rats dosed with ^{14}C-GTN but the percentage of GTN converted to glycerol was about 6% after 4 hr. The GMNs are probably water-soluble enough to be excreted faster than they can be metabolized further to glycerol. This suggestion is supported by the work of Needleman and Hunter (1965) who showed that GMN was practically unaltered by liver homogenates in vitro.

Metabolite Gradients Across the Lungs

The lungs were found to be sites of GDN metabolism in vivo and in vitro. Table 5.11 shows that lung homogenates were capable of metabolizing GTN to GDNs and then to GMNs. The work of Heinzow and Zeigler (1981) in which GDNs were detected in the non-recirculating effluent of lung perfusions containing GTN supports the contention that GTN is metabolized in the lung.

Plasma GMN profiles across the lung are variable showing no consistent trend in respect of their uptake or release (Figure 5.48(b)).

Metabolite Gradients Across the Liver

Portal vein - hepatic vein plasma GDN and GMN gradients show a consistent uptake of these compounds by the liver. This is in contrast to the gradients of GDNs and GMNs across the leg and lung which were sometimes an A-V gradient and other times a V-A gradient. This might suggest that the liver is the primary site of GDN and GMN metabolism while extra-hepatic tissues are the primary sites of GTN metabolism.

6.3.4. Effects of Glyceryl Dinitrates on Nitroglycerin Pharmacokinetics

In vitro experiments showed that the rate of metabolism of GTN in human erythrocytes and homogenates of sheep liver, lung, muscle, vena cava and aorta was reduced by the addition of GDNs. It was therefore decided to investigate whether GDNs were able to reduce the clearance of GTN *in vivo*. Bolus doses of GDNs administered during infusions of GTN had a slight effect on the arterial GTN plasma concentrations but a marked effect on the venous plasma GTN concentrations. Pharmacokinetic parameters of GTN in sheep administered GDNs were different to those of sheep not administered GDNs during the GTN infusion. GTN availability across the leg was increased by GDN administration and this was associated with a decrease in intrinsic clearance by the leg. The mean residence time of GTN in the leg was clearly increased by the administration of GDNs.

A more pronounced effect of GDNs on the pharmacokinetics of GTN is seen by plotting cumulative venous AUC/cumulative arterial AUC for GTN versus time (Figure 6.1). In Figure 6.1 it is seen that shortly after the administration of GDNs during GTN infusion the value of the

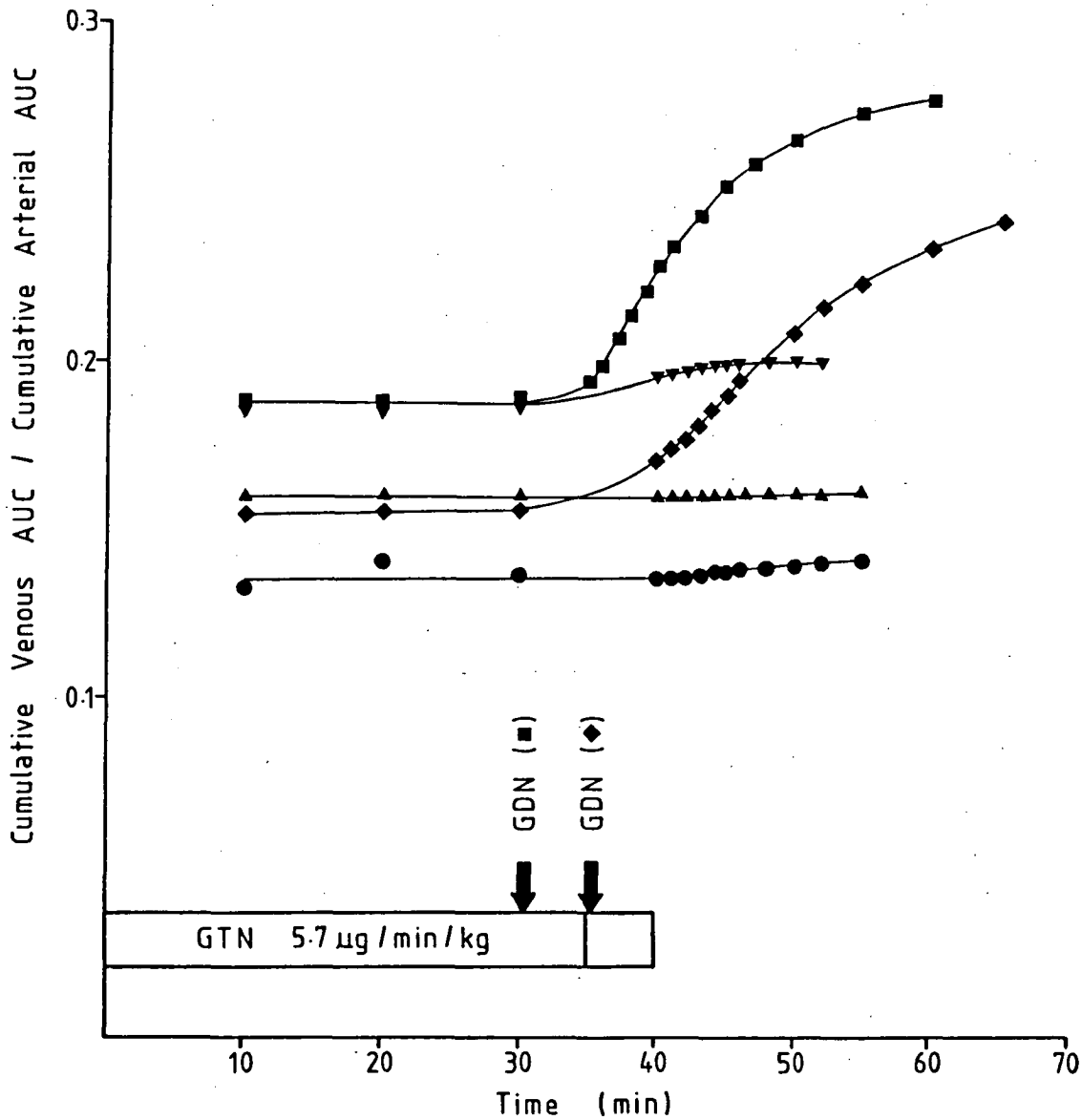


Figure 6.1 Effect of a bolus dose of GDNs on the ratio of cumulative venous and arterial AUCs in sheep receiving continuous intravenous infusions of GTN. Sheep 10, (■); Sheep 11, (●); Sheep 12, (▲); Sheep 29, (■) and Sheep 30, (◆).

ratio of cumulative AUCs begins to increase showing that the GDNs have a rapid onset of action in reducing the clearance of GTN. When the GTN infusion was stopped the cumulative AUCs ratio continued to increase at all times sampled thereafter. In sheep that did not receive a bolus dose of GDNs, the ratio of cumulative venous AUC/cumulative arterial AUC remained constant or increased only marginally (Figure 6.1).

These results are consistent with GDNs inhibiting the metabolism of GTN in the muscle and blood vessels of the leg. They also appear to account, at least in part, for the increase in availability of GTN across the leg and liver and the decreasing intrinsic clearances observed during infusions of increasing doses of GTN. Moreover, the increase in the half-lives of GTN elimination from arterial and venous plasma and the decrease in venous systemic clearance after serial bolus doses of GTN could be explained, in part, by the inhibition of GTN clearance by increasing amounts of GDNs formed from preceding bolus injections.

The finding that the arterial-venous gradient in GTN plasma concentrations was reduced at larger GTN infusion rates (as illustrated by the increased availability) is also consistent with the results of Armstrong-Moffat et al. (1981). Those workers found that after continuous administration of GTN to dogs for 5 days, the arterial-venous GTN gradient was abolished. They suggested that the disappearance of the gradient was possibly due to saturation at sites of GTN uptake. No mention was made of the rate of GTN administration used in that study but it is tempting to suggest that it was a relatively small rate (i.e., $<$ 1 µg/min/kg). In that event, the larger doses of GTN administered in the present work might have resulted in

the abolition of the arterial-venous GTN gradient in much less time than 5 days of continuous infusion. Indeed, the A-V gradient in sheep was abolished by serial bolus GTN doses (Figure 5.28) which were administered over a total of about 4 hr.

The abolition of the GTN concentration gradient at larger GTN infusion doses could be facilitated by the clearance of GDNs being reduced at larger GDN concentrations. It was seen that the half-lives of elimination of plasma GDN concentrations, formed during infusions of 0.4 μg GTN/min/kg (Figure 5.44) were 35 to 40 min (Table 5.19). It is possible that these half-lives could be considerably extended during chronic infusions of GTN, leading to accumulation of GDNs. Evidence for the concentration-dependent metabolism of GDNs comes from the data describing GDN metabolism in human erythrocytes (Table 5.10). Moreover, Table 5.9 shows that in blood, larger GDN concentrations inhibit GTN metabolism to a greater extent than smaller GDN concentrations. If these effects can be extrapolated to the muscle and other sites of tissue metabolism of GDNs then it is likely that the reason for the abolition of the arterial-venous GTN gradient on chronic GTN infusions (Armstrong-Moffat et al., 1981) is the inhibition of GTN metabolism in the leg by GDNs.

It has been shown very recently that the clearance of another organic nitrate drug, ISDN, in rats was reduced in the presence of the 5- and 2- mononitrate metabolites (Sutton and Fung, 1984). Those workers postulated that this interaction arose as a result of the mononitrates inhibiting the metabolism of the parent compound. A similar phenomenon has been reported for diazepam and desmethyldiazepam in humans (Klotz et al., 1976) and for lignocaine and its mono-N-deethylated

metabolite, monoethylglycinexylidide, in the isolated perfused rat liver (Pang and Rowland, 1977; Lennard et al., 1983).

6.4. PHARMACODYNAMICS OF NITROGLYCERIN IN SHEEP

Bolus GTN doses were found to reduce the MABP and MLVP in a dose-dependent manner. Bogaert and Rosseel (1972b) have previously reported similar effects (i.e., changes in hind-leg perfusion pressure) in the dog but did not quantify plasma GTN concentrations. In the current work, when plasma concentration and the AUCs for the both arterial and venous plasma were related to the reduction in MABP and MLVP, different slopes were obtained for the arterial and venous data. One explanation for the different slopes may be the A-V gradient in plasma GTN concentrations.

The dose dependency of duration of effect and the association of the larger GTN effects with a reduced cardiac output are consistent with the results of O'Rourke et al. (1971) with conscious dogs and De Maria et al. (1974) with humans. The reduction in cardiac output results from a reduction in left ventricular work as reflected by the MLVP. This reduction in cardiac output should be avoided when commencing GTN infusions in patients suffering acute myocardial infarction. In clinical practice GTN is often administered as a small IV bolus dose followed by a continuous infusion (Hill et al., 1981). If the bolus is delivered too quickly, the cardiac output may fall and exacerbate an already ischaemic myocardium (Parratt 1974; 1979).

Blood pressure responses to bolus doses of GTN in dogs and rabbits (Bogaert et al., 1970) were reported not to parallel the plasma levels of GTN. However, those workers used an indirect analytical method for plasma GTN levels which measured total organic

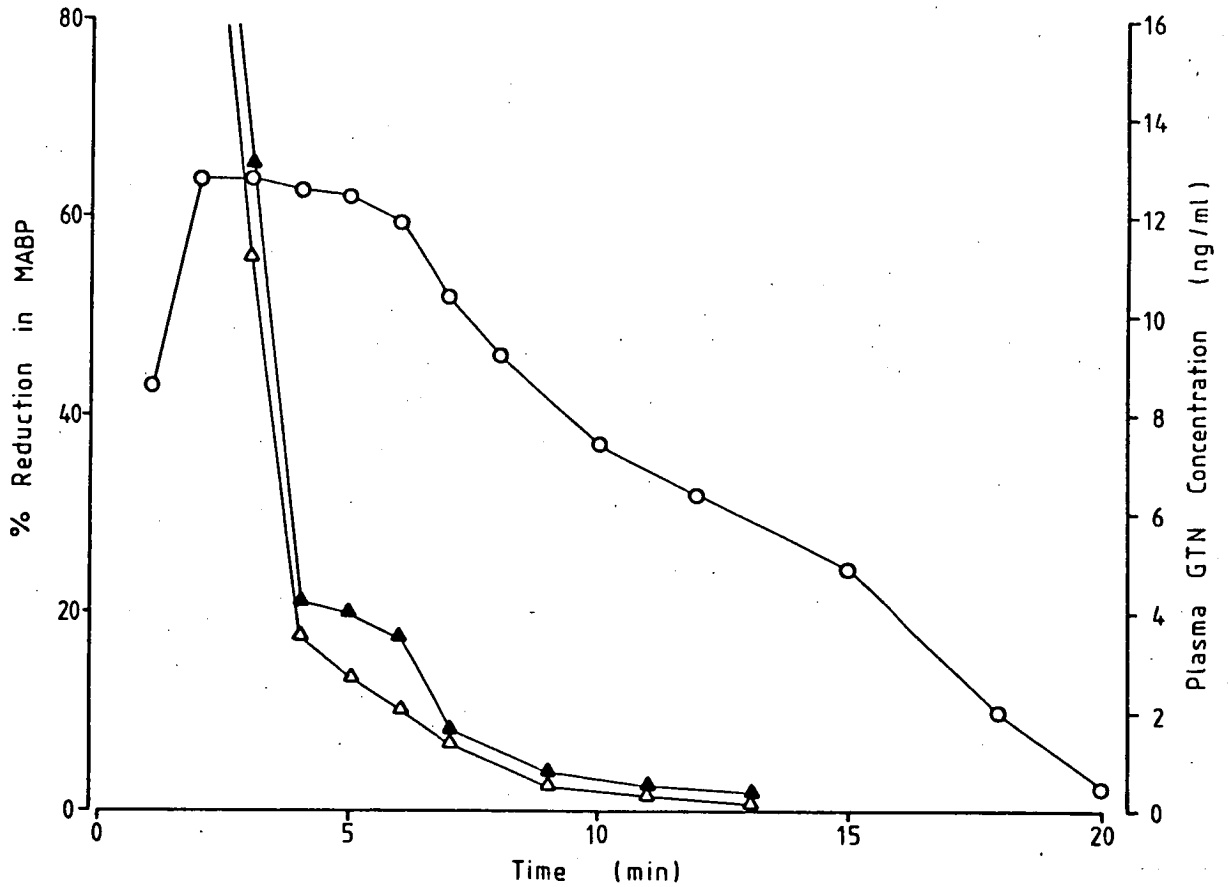


Figure 6.2 The temporal relationships of the reduction in mean arterial blood pressure (MABP) (○) and arterial (△) and venous (▲) plasma GTN concentrations elicited by a bolus dose of 54.5 µg GTN/kg in Sheep 13.

nitrate since no appropriate method to measure parent drug or separated metabolites was available at the time. Therefore, it is likely that Bogaert et al. (1970) were also measuring GDNs and GMNs. In the current work blood pressure appeared to return to baseline values in parallel with falling GTN plasma levels. An example of this is presented in Figure 6.2 which shows MABP returning to the baseline value at about the same rate as the detectable GTN venous and arterial plasma concentrations decay following a bolus GTN dose of 54.4 μg/kg into sheep No. 13.

Continuous IV infusions of GTN were associated with much less drastic hypotensive effects than the bolus doses provided. The rates at which GTN was administered in this study caused a modest dose-dependent decrease in MABP and a small dose-dependent increase in cardiac output, both effects of which are consistent with some previous studies (O'Rourke et al., 1971; Kovick et al., 1976). Moreover, the reduction in MABP was related to peak plasma GTN concentrations and AUCs in a dose-dependent manner. In the current work blood pressure fell as the GTN infusion commenced and a plateau was reached which coincided with steady-state plasma GTN levels. On termination of the infusion, blood pressure returned to baseline values in unison with the detectable plasma GTN concentrations. An example of this is presented in Figure 6.3. The results presented in this figure were obtained in experiments using a GTN infusion rate of 0.4 μg/min/kg. This rate of GTN administration is similar to that used by Armstrong et al. (1983) in congestive cardiac failure patients to study the relationship of arterial plasma GTN concentrations and the haemodynamic responses elicited by GTN (21 μg/min). Their results showed that as the plasma GTN concentrations increased to a steady-state

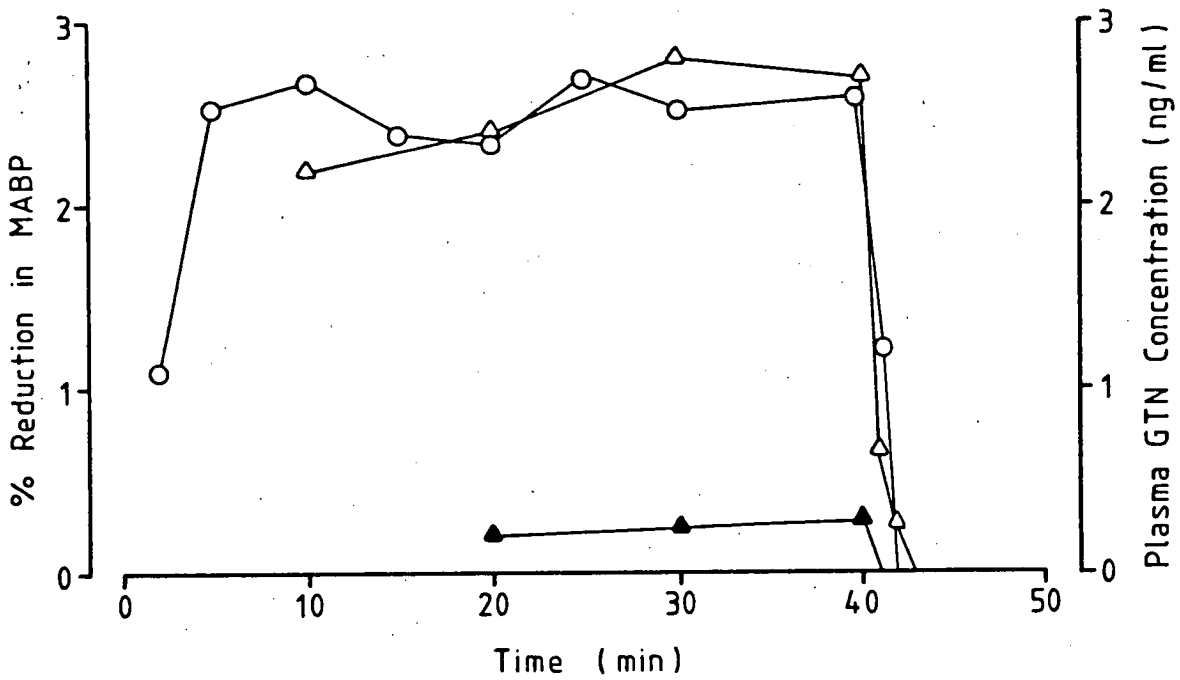


Figure 6.3 The temporal relationships of the reduction in mean arterial blood pressure (MABP)(○) and arterial (△) and venous (▲) plasma GTN concentrations elicited by a continuous intravenous infusion of GTN at a rate of 0.4 µg/min/kg in Sheep 16.

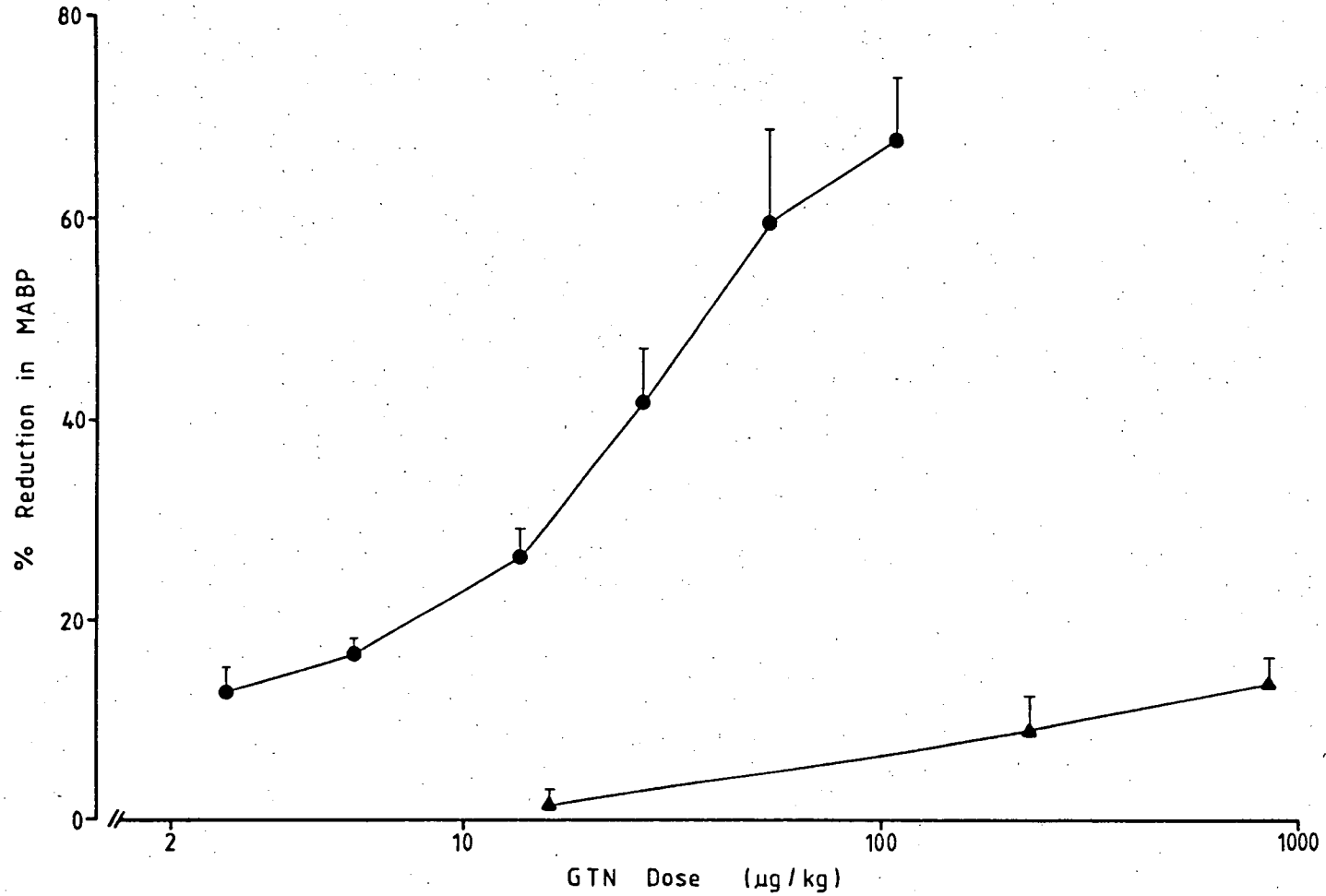


Figure 6.4 Comparison of the log GTN dose-response curves for the reduction in mean arterial blood pressure (MABP) evoked by GTN delivered as bolus doses (●) or as continuous intravenous infusions (▲) in sheep. Each point represents the mean ± se of the results for 3 sheep.

value of about 4 ng/ml, the pulmonary capillary wedge pressure gradually fell to a plateau which was 20% below baseline and which was maintained throughout the course of the infusion. Armstrong et al. (1983) reported very small changes in systolic blood pressure in their patients. The small changes in blood pressure are consistent with results obtained in the anaesthetized sheep. In summary, the results obtained in the anaesthetized sheep are in accord with the results of Armstrong et al. (1983) using cardiac failure patients, in that a maximal haemodynamic response occurred in parallel with maximal plasma concentrations.

A comparison of the effects of GTN administered by IV bolus and continuous IV infusion is seen in Figure 6.4. It is clear that a dose of GTN delivered by bolus will have a much greater hypotensive effect than the equivalent dose administered as a continuous infusion. Bogaert et al. (1970) also showed that the absolute amount of GTN administered was less important than a rapid change in the plasma GTN concentrations in eliciting a haemodynamic response to GTN.

The depression of arterial pressure by GTN is augmented in anaesthetized open chest preparations (Vatner and Heyndrickx, 1975). Therefore, the degree and duration of MABP reduction induced by bolus and continuous doses of GTN in the current work may have been greater than would have been recorded in conscious animals. This is because in the open chest anaesthetized animal the reflex control of the circulation is attenuated (Vatner and Heyndrickx, 1975). Added to this is the hypotensive effect of pentobarbitone, the anaesthetic agent used in this work.

6.5. CLINICAL IMPLICATIONS OF NITROGLYCERIN STUDIES

6.5.1. Therapeutic Monitoring

The results from the present work show that the interpretation of plasma GTN concentration data must be in relation to: (i) possible degradation of GTN after blood sample collection; (ii) the site of blood sample collection; and (iii) the effect of metabolites of GDN. A previous study (Cossum et al., 1978) has shown the potential for intravenous administration systems to influence the plasma GTN concentrations due to reduced availability of GTN.

The current studies have also pointed out a potential for incorrect GTN dosage adjustments to be made. When GTN infusions are administered over extended periods of time the possibility exists that venous plasma GTN concentrations will slowly increase, possibly due to GDN-induced reduction in GTN metabolism. Thus, a reduction in GTN dosage may be instituted. The oral bioavailability of GTN is markedly enhanced in patients with certain liver diseases (Porchet and Bircher, 1982). Nevertheless, the current results show that the liver is probably not the major site of GTN clearance. Thus, reduced dosing of GTN to patients with liver disease may not be necessary.

When GTN is administered intravenously it is mandatory to monitor haemodynamic responses. The present results adequately display the rapid changes in blood pressure and cardiac output which can accompany the rapid IV administration of GTN. It is therefore likely that clinicians would rely mostly on haemodynamic responses to GTN rather than in plasma GTN concentration data. The present results serve to reinforce that practice.

6.5.2. Tolerance

Two of the current hypotheses put forward to explain the mechanism of action of organic nitrates in relaxing vascular smooth muscle require the GDNs to be formed prior to or subsequent to activation of the smooth muscle receptor by GTN. The present work has shown that GTN transformation to GDN can be inhibited by GDN itself. This suggests that the relaxation elicited by GTN may be reduced or prevented by GDNs.

Tolerance to the haemodynamic effects of organic nitrates, according to the hypothesis of Needleman and Johnson (1973), results from there being less sulphydryl groups on receptors available to interact with the parent organic nitrate. Alternatively, the hypothesis of Ignarro et al. (1981) suggests that organic nitrates are reduced within vascular smooth muscle and the nitrite ion so formed elicits a response by reacting through an active intermediate, S-nitrosothiol. Tolerance may be associated with a reduced formation of the active intermediate.

The results obtained in the current work may provide an important link between tolerance development to organic nitrates and altered pharmacokinetics during long-term administration of those drugs. The development of tolerance to GTN may be as a result of the reduced availability of sulphydryls (Needleman and Johnson, 1973) or an active intermediate, or by inhibiting the reaction of the active metabolite with the receptor (Ignarro et al., 1981). The former possibility may be caused by the accumulation of GDNs in the smooth muscle cells, interfering with the reaction of GTN and sulphydryls. It is known that the GDNs have only 1/15 - 1/20 of the vasodepressive potency of

GTN (Needleman et al., 1969; Bogaert et al., 1968). When they are in abundance in the vascular cell they may prevent the interaction of GTN with the receptor either by exclusion or by reacting with the receptor at a different site to GTN and which prevents GTN reacting with the receptor. It is likely that GDNs are metabolized to GMNs in the vascular tissue (although none was detected in the in vitro experiments of the current study, probably because of the small vascular tissue masses used). The GMNs may also accumulate upon the eventual degradation of the GDNs during chronic GTN therapy and prevent GTN from reacting with the receptor. GMNs are known to lack vasodilator activity (Needleman et al., 1969).

Increasing metabolite concentrations in the vascular cells may also prevent the formation of the active intermediate proposed in the hypothesis of Ignarro et al. (1981) by reducing the conversion of the pre-cursor, nitrite ion, from GTN. Alternatively, if tolerance develops as a result of some interference in the interaction of the active intermediate with the receptor, then accumulating metabolite levels may be responsible.

Evidence of tolerance development to the hypotensive effects of GTN during long-term low dose GTN therapy in humans is conflicting (Abrams, 1980). However, tolerance to GTN has been described in rats, rabbits and dogs, with the degree of tolerance directly related to the dose of GTN administered (Abrams, 1980). More information is available on the tolerance developed to ISDN. Tolerance to high dose chronic oral ISDN therapy (up to 480 mg ISDN daily) in humans develops within 1-2 days (Thadani et al., 1980). The same group of workers later reported reduced plasma clearance of ISDN at the larger chronic doses used in their initial study (Fung et al., 1981). Fung and

Parker (1983) reported the presence of a previously undetected prolonged ISDN disappearance phase after 60 mg ISDN orally 4 times a day for up to 3 weeks. The terminal half-life of 7.7 hr for ISDN was slower than for the terminal half-lives of the 2-ISMN and 5-ISMN metabolites which were 3 hr and 6 hr, respectively. These results suggested that the metabolites were inhibiting the clearance of ISDN, a point which was later verified by Sutton and Fung (1984). Other workers have reported that 5-ISMN accumulates on chronic dosing of ISDN (Shane et al., 1978; Bruyneel et al., 1982).

Recent evidence linking increased levels of organic nitrate metabolites with the occurrence of tolerance to the hypotensive effects of the parent organic nitrate comes from the studies of Blasini et al. (1982) and Kogi et al. (1982), both of which are cited in a recent review by Rietbrock and Woodcock (1984). Dosing of patients with ISDN 40 mg 4 times daily for 7 days resulted in the complete attenuation of the hypotensive effects of ISDN (Blasini et al., 1982). However, 36 hr after the final ISDN dose another dose of 40 mg ISDN had its original maximal hypotensive effect on blood pressure. During the 36 hr break from ISDN dosing, 5-ISMN would have almost disappeared from the plasma given its half-life of about 5-6 hr (Abshagen and Sporl-Radun, 1981; Fung and Parker, 1983).

Kogi et al. (1982) appeared to overcome the problem of tolerance development to ISDN in dogs by administering the drug from a transdermal preparation and it was suggested the reason for this was the much lower ISMN plasma concentrations arising in the plasm after transdermal dosing of ISDN compared to the large ISMN plasma concentrations produced following oral administration of the drug.

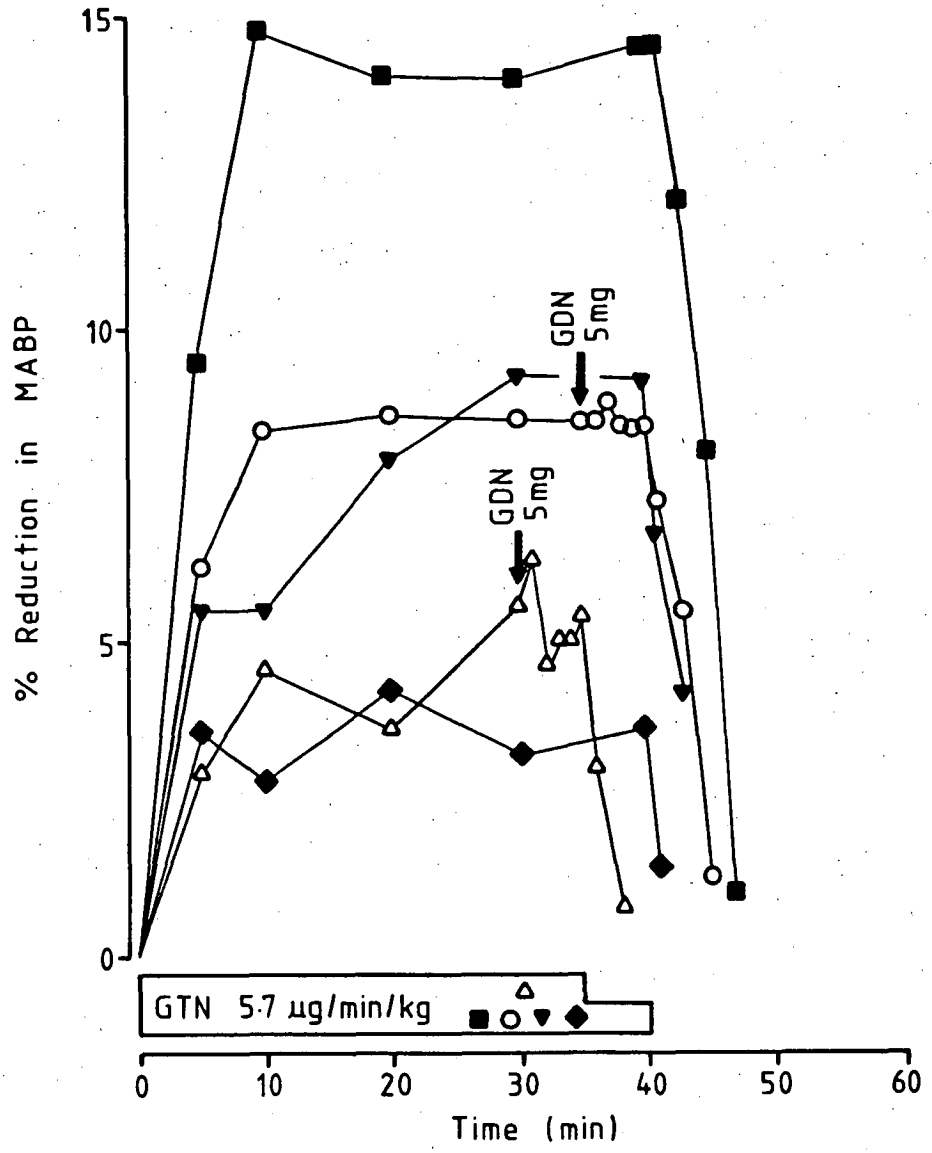


Figure 6.5 Effect of adding a 5 mg bolus dose of GDNs on the mean arterial blood pressure in sheep infused with 5.7 µg/min/kg GTN. Open symbols represent sheep receiving bolus dose of GDNs and closed symbols sheep not receiving bolus.

Similar experiments to those just described need to be performed using GTN to be able to determine a role for GDNs in the mechanism of tolerance development to the hypotensive effects of GTN. A diminution of the hypotensive effects of GTN was not observed when bolus doses of GDNs were administered during GTN infusions in sheep (Figure 6.5). One explanation for this could be that the dose of GDNs administered as a bolus (5 mg), although inhibiting GTN metabolism at the vascular receptor, was large enough to exert a substantial hypotensive action of its own despite the GDNs having only about 1/15 of the vasodepressive action of GTN. Experiments designed to elucidate the role of the GTN/GDN interaction in the development of tolerance to GTN will need to incorporate relatively small GDN doses.

In summary, pharmacokinetic analysis of the organic nitrates may have given some insight into the cause of tolerance to the hypotensive effects of these drugs. Perhaps the most important and most difficult aspect to elucidate is whether organic nitrate metabolites can be proven to interfere with the interaction of the parent organic nitrate and its (possible) receptor in the vascular smooth muscle.

6.6. PHARMACOKINETICS OF ASPIRIN IN SHEEP

There are very few reports in the literature concerning IV dosing of ASA. Bolus ASA intravenous doses have been administered to dogs (Leonards, 1962) and humans (Rowland and Riegelman, 1968; Loo and Riegelman, 1968), and continuous IV infusions of ASA have been delivered to humans (Loo and Riegelman, 1968; Harris and Riegelman, 1969). These studies demonstrated that the delivery of ASA to the systemic circulation was dependent on the route of administration. Harris and Riegelman (1969) infused ASA into the portal vein and vena

cava of dogs and compared the resulting ASA AUCs based on aortic plasma concentration profiles with AUCs obtained in aortic plasma after an equivalent oral dose of ASA. This work demonstrated significant ASA extraction across the liver and gut wall. The present work extends these findings to demonstrate significant extra-hepatic metabolism of ASA and an *in vivo* interaction of ASA and SA.

6.6.1. Plasma Protein Binding

Plasma protein binding studies *in vitro* showed that the fraction of ASA unbound in normal sheep plasma for ASA concentrations of 10 and 50 µg/ml was about 0.35 - 0.37. Given that ASA binding is dependent on the ASA concentration and the plasma protein concentration (Aarons et al., 1980) the values of f_u for ASA at ASA concentrations of 10 and 50 µg/ml are consistent with the results of Aarons et al. (1980). Those workers reported a f_u for ASA of 0.32 (in the absence of SA) at an ASA concentration of 0.2 µg/ml and a protein concentration of 40 mg/l.

The presence of SA at a concentration of 50 µg/ml increased the free fraction of ASA to about 0.49 and this is consistent with the displacement of ASA from human and bovine albumin by SA as reported previously by Aarons et al. (1980). The free fraction of SA in normal sheep plasma was 0.15 - 0.22 for SA concentrations of 10 and 50 µg/ml (Table 5.6). These values are close to the values of f_u for similar SA concentrations in dog and horse plasma (Sturman and Smith, 1967).

During anaesthesia and surgery, the plasma albumin concentrations of all sheep fell. This decrease during surgery is probably a result of an increased catabolism of albumin and/or a greater distribution of

albumin into the extravascular space (Davies, 1976). The ramification is that the protein binding of ASA and SA would be altered.

In in vitro studies on the binding of ASA and SA, diluted plasma (1:1) increased the f_u of ASA with and without added SA relative to normal plasma whereas potassium fluoride and pentobarbitone had a negligible effect on the binding of ASA. The results of ASA and SA protein binding determinations in samples taken during ASA infusions are consistent with the in vitro findings and results reported in other studies (Moran and Walker, 1968; Spector et al., 1972; Aarons et al., 1980). Thus, the major determinants of ASA binding in sheep plasma were:

(i) albumin concentration, and

(ii) SA concentration.

6.6.2. Arterial-Venous Plasma Aspirin Gradients

Extraction Across the Leg

The mean availability of total and free ASA across the hind leg of the sheep during ASA infusions at 485 μg/min/kg was 0.86 and about 0.81, respectively. Results from the in vitro experiments showed that ASA is hydrolyzed to SA when in contact with muscle tissue homogenates and so the observed arterial-venous ASA concentration gradient is probably a result of ASA hydrolysis in the leg tissues. The rate of ASA hydrolysis by muscle homogenates was not dependent on the initial ASA concentration nor was SA able to inhibit the metabolism of ASA.

These results suggest that muscle tissue could be a site of ASA clearance which contributes to the relatively short half-life of ASA in vivo. Although it has been recorded that ASA is hydrolyzed to SA

in the gastro-intestinal tract (Rowland et al., 1972), blood (Rowland and Riegelman, 1967), liver (Harris and Riegelman, 1969), kidney and other organs (Rainsford, 1984) by the so-called "aspirin esterases," it has not previously been reported that muscle tissue has aspirin esterase activity. ASA may acetylate the proteins of muscle since Rainsford et al. (1983) and Rainsford (1984) report that ASA is capable of acetylating a variety of proteins.

Extraction Across the Lungs

Despite the in vitro experiments showing that lung tissue homogenates can hydrolyze ASA to SA, the mean availability of ASA across the lungs during in vivo experiments was close to unity (Table 5.20). The apparent lack of extraction may arise from either:

(i) ASA not being distributed into the lung cells where the esterases are likely to reside; or

(ii) as a consequence of the enormous flow rate of blood (the lungs receive the entire cardiac output).

According to the negligible well stirred model, the availability (F) is given by the equation:

$$F = \frac{1}{1 + f_u \, Cl_{int}/Q} \qquad (6.2)$$

where f_u is the fraction unbound, Cl_{int} is the intrinsic clearance, and Q the blood flow. If the flow rate Q is very much greater than $f_u \cdot Cl_{int}$ (as is likely in the lung), the availability will appear to be unity.

Extraction Across the Liver

The results of the current study are consistent with earlier reports which showed that the liver is responsible for substantial degradation of ASA. Harris and Riegelman (1969) showed that the mean availability of ASA infused into the portal vein was only 0.64 of the availability of ASA infused into the vena cava of conscious dogs. Results for the anaesthetized sheep preparation show that the mean availability of ASA across the liver during infusions was 0.72 - 0.77.

6.6.3. Arterial-Venous Salicylic Acid and Salicyluric Acid Gradients

Metabolite Gradients Across the Leg

The arterial-venous gradient in total and free plasma SA concentrations was reversed after the termination of the ASA infusion at 485 µg/min/kg. Because SA was still being formed at the end of the blood sampling after the infusion of ASA at 485 µg/min/kg no plasma AUCs could be calculated and therefore no estimate of availability was possible. However, the mean SA availability across the leg could be calculated from the data obtained for the infusion of ASA at 61 µg/min/kg and was found to be 1.19 (Table 5.23) indicating that SA was not being further metabolized in the leg muscle. An availability greater than unity would arise from the production of SA in the leg on ASA metabolism.

The above conclusions are consistent with the *in vitro* findings. In the *in vitro* incubations of ASA with muscle homogenates, ASA was metabolized and could be totally accounted for by the production of SA. Metabolism of SA involves hydroxylation or conjugation with glycine or glucuronic acid. As there is no evidence for these path-

ways in skeletal muscle (Gram, 1980), it is not surprising that SA is not metabolized during its passage across the leg.

Bolus doses of SA delivered into the aorta resulted in a venous-arterial gradient in SA concentrations. The rate of elimination of SA was considerably slower after the bolus dose of 1200 mg SA than after the lesser SA doses. This finding is consistent with previously published results which show the elimination of SA to be non-linear in nature as a result of saturation of its major metabolic pathway (Levy, 1965; Tsuchiya and Levy, 1972).

Total and free SU concentrations were typically slightly greater in the arterial plasma than venous plasma during the early stages of their formation but the gradient had been abolished or reversed by the time the ASA infusion was terminated. Administration of the 1200 mg bolus SA dose temporarily resulted in the formation of an arterial-venous gradient in SU concentrations.

Metabolite Gradients Across the Lungs

The mean availability of SA across the lungs was close to unity and therefore comparable with the values for ASA for the two ASA infusion rates. Glucuronide conjugation occurs in mammalian lung tissue but glycine conjugation does not (Gram, 1980) and so it is possible that SA could be further metabolized to salicyl acyl glucuronide or salicyl phenolic glucuronide. However, as suggested earlier for ASA (6.6.2.), distribution of SA in the lung and the high pulmonary blood flow may be such to result in an availability close to unity.

Metabolite Gradients Across the Liver

SA has a moderately low hepatic extraction ratio (Rowland and Tozer, 1980). In the sheep, the availability of SA across the liver was calculated to be 0.91, i.e., an extraction ratio of 0.09. The very low extraction ratio results in part from SA generated in the liver during ASA metabolism. Leonards (1962) reported that after oral administration of ASA to dogs, plasma ASA concentrations in the hepatic vein were less than those in the portal vein, a result consistent with the findings of the current work. Leonards (1962) also reported that plasma salicylate concentrations in the hepatic vein were larger than in the portal vein. The large extraction ratio for ASA and its conversion to SA would lead to an availability of SA greater than unity. An availability exceeding unity may also be due in part to the analytical method used by Leonards (1962) who used the method of Brodie et al. (1944). This method measures total salicylate, (i.e., salicylate, salicylurate and salicylate glucuronides) after vigorous hydrolysis.

The present results show that SU concentrations in the hepatic vein were greater than in the portal vein which is consistent with its formation in the liver. In humans SU production is mainly via hepatic metabolism and renal metabolism is a minor pathway (Schachter and Manis, 1958; Lowenthal et al., 1974). The proportions of renal and hepatic production of SU in sheep are unknown.

6.6.4. Effect of Salicylic Acid on Aspirin Pharmacokinetics

In order to investigate whether SA administration during ASA infusion had any effects on ASA pharmacokinetics ASA infusions at 61 µg/min/kg were used. This was because SA formed during ASA

infusions at 485 μg/min/kg achieved plasma concentrations of the order of 50 μg/ml. It was intended to infuse SA as bolus doses into sheep which had easily detectable ASA plasma concentrations yet which had only small SA plasma concentrations. This was necessary to monitor the effects of a dose of SA on the clearance of ASA.

SA had no discernible effects on the elimination of ASA except for the 1200 mg bolus dose of SA when the half-life of elimination of ASA was approximately two-thirds that obtained for lower SA doses. This effect was observed for both total and free ASA plasma concentrations and was probably due to the displacement of ASA from plasma proteins by SA. In vitro protein binding studies of the interaction of ASA and SA showed that SA could displace ASA from protein binding sites, a result which is consistent with the in vitro findings of Aarons et al. (1980). ASA has been classified as a drug of intermediate hepatic extraction ratio (Rowland and Tozer, 1980). Since hepatic clearance is a function of intrinsic clearance and f_u, the change in the total clearance of ASA is affected by a change in f_u. It is unlikely that SA affected the hepatic intrinsic clearance of ASA as in vitro experiments failed to show any inhibition of ASA metabolism in liver (or muscle or lung) homogenates by SA. As bolus dosing of SA had no effect on cardiac output the increased elimination of ASA after the 1200 mg SA dose appears to be due solely to the effects of SA on ASA binding.

6.7. PHARMACOKINETICS AND PHARMACODYNAMICS OF ASPIRIN IN MAN

6.7.1. Plasma Aspirin Levels

The peak ASA levels following ingestion of 300 mg soluble or micro-encapsulated ASA in the current study were generally in agreement with other workers' reports. Rowland et al. (1972) and Rance et al. (1975) reported peak plasma ASA levels after 650 mg soluble ASA as 10-23 μg/ml and a mean of about 10 μg/ml, respectively. The mean peak plasma ASA concentrations after 300 mg soluble ASA in the current work were about 3.5 μg/ml.

The slow-release ASA used in the current work was in the form of micro-encapsulated granules and is a relatively new product. The differences in bioavailability of ASA from single doses of the soluble and similar slow release formulations are consistent with results presented by Brantmark et al. (1982), and Ross-Lee et al. (1982).

The approximate direct relationship for (detectable) peak ASA concentrations and doses of the slow release ASA formulation (Table 5.24) is consistent with the direct relationship between steady-state plasma ASA concentrations and dose after IV infusions in sheep (Figure 5.61), and with the lack of dose dependency of ASA kinetics reported by Pedersen and Fitzgerald (1984) after oral doses of ASA in man.

6.7.2. Aspirin Bioavailability and Platelet Function

The degree of inhibition of platelet function was similar for the soluble and slow-release ASA formulations despite marked differences in plasma ASA concentrations. The magnitude of platelet function inhibition is unrelated to the peak ASA concentrations or to the area

under the plasma ASA concentration-time curve. A possible explanation for this lack of correlation is the irreversible and saturable acetylation of platelets (Roth et al., 1975). From the slow-release formulation the plasma ASA concentrations were very low, and in two subjects were undetectable ($<$ 0.1 µg/ml). However, there was a corresponding marked inhibition of platelet function. It is apparent, therefore, that platelets may be acetylated when plasma ASA concentrations are very low. The lack of correlation between inhibition of platelet function and plasma ASA concentrations shows that plasma ASA concentrations cannot be used as an index of platelet inhibition produced by ASA.

The present study shows that low doses of either a soluble or a slow-release formulation are adequate for the inhibition of platelet function as assessed in these experiments. The dose of the slow-release formulation required to produce 50% of maximal inhibition of platelet aggregation/MDA production was less than 50 mg for AA and adrenalin, and less than 100 mg for collagen. This dose is comparable with doses reported for faster releasing ASA formulations. Burch et al. (1978) found that repeated daily administration of 20, 80 and 325 mg ASA for 5-7 days produced 61, 86 and > 95% inactivation of cyclooxygenase, respectively. Lorenz et al. (1981) found that repeated daily administration of 100 mg ASA almost totally inhibited aggregation and TXB_2 formation after induction by AA, adenosine diphosphate and low collagen concentrations. Larger collagen concentrations (of the order used in this study and in that of Masotti et al., 1979) resulted in reduced platelet aggregation for doses of 100 mg ASA per day but with complete suppression of TXB_2 formation (Patrano et al., 1980). The incomplete inhibition by ASA of platelet

aggregation induced by larger collagen concentrations probably results from collagen inducing aggregation by other mechanisms independent of TXA_2 synthesis (Emms et al., 1982). Relatively high doses (1,200 - 1,300 mg) of soluble ASA were employed in the initial clinical trials evaluating the efficacy of ASA for transient ischaemic attacks (Fields et al., 1977; The Canadian Co-operative Study Group, 1978). More recent studies have suggested that lower doses of soluble ASA may be preferred to the higher doses because the lower doses of ASA may inhibit platelet TXA_2 production and platelet aggregation without markedly interfering with PGI_2 production by vessel wall cyclo-oxygenase (Masotti et al., 1979; Preston et al., 1981; Hanley et al., 1981; Patrignani et al., 1982; Fitzgerald et al., 1983). This aspect is discussed in Section 6.8.

The effects of ASA on the in vivo endothelial cell wall production of PGI_2 per se are difficult to estimate because PGI_2 is a local hormone present only in very low concentrations in systemic plasma (Haslam and McGlenaghan, 1981; Blair et al., 1982). The metabolites of PGI_2 have been quantified (particularly 6-oxo-$PGF_{1\alpha}$) in the urine of subjects taking soluble ASA (Fitzgerald et al., 1983; Patrignani et al., 1982). Patrignani et al. (1982) suggested that 0.45 mg ASA/kg (i.e., about 30 mg) may be a suitable dose since platelet activity was impaired while renal production of PGI_2 was not during one month of therapy in healthy subjects. However, Fitzgerald et al. (1983) using urine levels of metabolites of TXA_2 and PGI_2 as indices of platelet and vessel wall cyclooxygenase activity found that even doses as low as 20 mg per day for 8 days had a marginal effect on PGI_2 production, the degree of selectivity decreasing with increasing dose.

Other workers have evaluated the effects of ASA on PGI_2 production by different techniques. Masotti et al. (1979) suggested that a single dose of approximately 175 mg (2.5 mg/kg body weight) had no effect on PGI_2 synthesis induced by forearm ischaemia, whereas larger doses (3.5 to 10 mg/kg body weight) significantly inhibited PGI_2 synthesis with full recovery by 48 hr. Preston et al. (1981) found that 150 and 300 mg ASA substantially depressed the production of 6-oxo-$PGF_{1\alpha}$ from autologous human venous biopsies. Hanley et al. (1981) found 81 mg ASA taken 14 hr preoperatively resulted in approximately 60% depression of the release of PGI_2-like material from autologous postoperative venous biopsies. However, a single dose of 40 mg had not effect on PGI_2 synthesis by venous tissue.

6.8. CLINICAL IMPLICATIONS OF ASPIRIN STUDIES

As the ASA dose-platelet function response curves appear to be similar for the slow-release formulation examined in this study and a conventional soluble ASA formulation, the relative extent to which these formulations may affect vessel wall cyclooxygenase may be of clinical significance. The slow-release preparation in doses in excess of 100 mg produced maximum inhibition of platelet function via platelet cyclooxygenase as measured by *in vitro* methods. The extent to which the vessel wall cyclooxygenase was inhibited and the relationship of dose of ASA and inhibition of vessel wall cyclooxygenase was not determined in these studies. However, it is possible that the lower plasma ASA concentrations found for the slow-release formulation could be associated with a lesser inhibition of vessel wall cyclooxygenase than of platelet cyclooxygenase. A slow-release formulation may result in selective inhibition of platelet cyclooxygenase in the

portal circulation with only very small amounts of ASA being available systemically due to the significant hepatic first-pass effect for ASA. Thus, production of the anti-aggregating and vasodilator PGI_2 may be relatively unaffected.

Recent studies by Weksler et al. (1985a,b) have suggested that doses of ASA in the range 20-40 mg daily may be beneficial for patients with recent cerebral ischaemia and patients with atherosclerosis. The basis of this claim is that low ASA doses have a similar anti-platelet effect to high doses but they also have a cumulative anti-platelet effect. Moreover, Weksler et al. (1985b) found that those low doses of ASA produced marked inhibition of platelet aggregation and thromboxane synthesis in patients with coronary artery disease but only a partial depression of vascular PGI_2 production, as measured in vessel segments removed during surgery. Pedersen and Fitzgerald (1984) are others who have shown that oral doses of 20-40 mg ASA produce marked inhibition of thromboxane formation and they have also suggested that pre-systemic acetylation of platelet cyclooxygenase can be achieved by small doses of a conventional ASA formulation, but that slow administration of very low doses of ASA may offer an even better means of selective platelet cyclooxygenase inhibition.

One factor which may make the attainment of a selective pre-systemic acetylation of platelet cyclooxygenase more difficult than currently envisaged involves the significant extra-hepatic metabolism of ASA. As shown in the sheep experiments, extraction of ASA occurred across the leg of the sheep and this results from metabolism of ASA to SA. These results indicate that metabolism of ASA by the liver after oral administration only partly accounts for the observed availability

in man (Rowland et al., 1972; Pedersen and Fitzgerald, 1984). This availability is obtained when the availabilities of the liver, lung and leg are multiplied as is customary for organs in series (Gibaldi and Perrier, 1982). This suggests that the trans-hepatic gradient of ASA would not be as pronounced after an oral dose compared to not taking the extra-hepatic metabolism of ASA into consideration. It is possible that after very small oral doses of ASA the extraction of ASA by the liver would leave insufficient amounts of ASA to be available to the cyclooxygenase of the systemic vessel walls, regardless of extra-hepatic ASA metabolism. Nevertheless, this aspect of ASA metabolism needs to be borne in mind when designing low dose ASA protocols to try to achieve selective effects of ASA on cyclooxygenase.

Cerletti et al. (1981) have suggested that ASA and SA compete for binding sites on cyclooxygenase and that SA may protect the vessel wall cyclooxygenase more than platelet cyclooxygenase from the effects of ASA. Following administration of 300 mg slow-release ASA formulation, the peak plasma ASA/SA concentration ratio was about 0.1. In contrast, the ratio following administration of the 300 mg soluble formulation was about 0.3 which is similar to the value of 0.5 found by Rowland et al. (1972). SA had no effect on extraction or mean residence time of ASA in sheep but did displace ASA from plasma proteins at high concentrations of SA. It is possible that SA could also displace ASA from vascular cyclooxygenase yielding an apparent protective effect for SA in relation to the inhibition of PGI_2 production induced by ASA. In this respect a slow-release ASA formulation may be more beneficial because of the relative ASA and SA systemic blood concentrations arising from this preparation. Only when PGI_2

production is measured after administration of the slow-release ASA preparation can this aspect be elucidated.

The full clinical implications of this work await the reports of large-scale, international studies currently underway which are investigating the most appropriate dose, dosing interval and formulation of ASA to reduce the incidence of cerebro/cardiovascular disease. Judging by the results presented in this thesis and a survey of the current literature, it would be anticipated that ASA from a slow-release preparation would have, at least, an equivalent effect to an equivalent dose of ASA from a faster releasing preparation if, indeed, chronic ASA therapy is found to reduce the incidence of cerebro/cardiovascular disease.

6.9. CONCLUSIONS

This thesis has presented results which show that GTN and ASA are subject to significant extra-hepatic metabolism in sheep. Moreover, the disposition of both drugs in sheep is affected, to various degrees and in different manners, by their major metabolites. The full clinical ramifications of these findings await further investigations in man.

One aspect not covered in this thesis is the possibility of an interaction between ASA and GTN. Preliminary reports from some investigators suggest that the haemodynamic responses to GTN are attenuated by co-administration of ASA. Other reports suggest that ASA augments the haemodynamic responses to GTN. This is potentially a very important interaction considering the probable large numbers of patients dependent on sufficient haemodynamic response to GTN who use ASA either chronically or intermittently.

Despite the widespread use of GTN and ASA over many years, it is apparent that many avenues of research remain to be followed to more fully understand the pharmacokinetics and pharmacodynamics of these two important and interesting drugs.

APPENDIX

Table A1 — Intra- and Inter-Subject Variability in Control Platelet Aggregation Responses to Adrenalin

Subject	Day	% Light Transmission 1	2	3	4	5	MEAN	S.D.	S.E.	% C.V.
KS		15	16	14	65	21	26.2	21.8	9.7	83.4
EM		94	80	72			81.8	11.3	6.5	13.9
DK		15	36	12	82	15	32.2	29.7	13.3	92.2
SS		79	86	77			80.0	4.5	2.6	5.6
DR		83	86	90	82	84	85.2	3.1	1.4	3.6
HE		82	62	82			75.2	11.7	6.6	14.0
SG		76	87	86	82	79	82.0	4.8	1.7	5.8
MW		87	86	51			74.7	20.6	12.1	27.6
RH		83	85	82	82	16	69.9	29.8	10.6	42.7
AU		83	87	83			84.5	2.6	1.5	3.1
MS		-	81	82	84	85	83.7	1.5	0.9	1.9
SJ		12	87	15			38.0	42.9	25.2	113.0
WM		24	76	80	77	80	67.2	24.4	8.7	36.0
KN		77	85	85			82.3	4.6	2.7	5.6
JC		72	80	80	80	19	67.2	26.9	9.6	40.2
HW		12	30	21			21.3	8.7	5.2	41.0
MEAN		59.9	72.1	63.4	79.3	49.9				
S.D.		32.8	23.3	29.7	6.1	34.3				
S.E.		8.5	5.8	7.4	2.2	12.2				
% C.V.		54.8	32.4	46.9	7.7	68.8				

Total Population Variability

Mean = 63.4
S.D. = 29.3
S.E. = 3.4
% C.V. = 46.0

Table A2 — Intra- and Inter-Subject Variability in Control Platelet Aggregation Responses to Collagen

Subject	Day	% Light Transmission 1	2	3	4	5	MEAN	S.D.	S.E.	% C.V.
KS		86	82	80	82	83	82.6	2.2	1.0	2.7
EM		90	85	82			85.7	4.0	1.9	4.7
DK		90	91	87	82	82	86.8	4.1	1.9	4.8
SS		86	90	82			86.3	3.7	2.2	4.3
DR		90	90	85	84	85	86.8	2.9	1.3	3.4
HE		92	82	83			86.0	5.6	3.3	6.6
SG		81	86	77	78	80	80.8	4.0	1.8	4.9
MW		91	92	87			90.3	2.5	1.5	2.8
RH		80	86	80	81	83	82.3	2.7	1.2	3.3
AU		92	90	82			88.1	5.1	3.0	5.8
MS		-	87	85	85	82	84.7	2.5	1.2	2.9
SJ		90	87	84			87.1	3.0	1.7	3.4
WM		83	77	85	82	82	81.7	3.1	1.5	3.9
KN		84	92	85			87.0	4.3	2.5	5.0
JC		78	82	85	82	84	83.3	4.2	1.9	5.1
HW		85	86	90			87.0	2.6	1.5	3.4
MEAN		86.6	86.8	83.8	82.0	82.7				
S.D.		4.6	4.3	3.1	2.0	1.5				
S.E.		1.2	1.0	0.8	0.7	0.5				
% C.V.	—	5.3	5.0	3.7	2.5	1.9				

Total Population Variability

Mean = 84.7
S.D. = 3.4
S.E. = 0.4
% C.V. = 4.0

Table A3 — Intra- and Inter-Subject Variability in Control Platelet Aggregation Responses to Arachidonic Acid

SUBJECT	DAY	1	2	3	4	5	MEAN	S.D.	S.E.	%C.V.
KS		80	82	83			82.1	2.0	1.1	2.4
EM		80								
DK		83	87	85			85.3	2.0	1.1	2.3
SS		86								
DR		86	80	-			83.2	-	-	
HE		83								
SG		82	80	80			80.5	0.8	0.5	1.0
MW		82								
RH		85	82	-			83.7		-	
AU		83								
MS		82	85	87			84.8	2.2	1.3	2.6
SJ		75								
WM		82	79	82			80.5	1.3	0.7	1.6
KN		90								
JC		82	80	82			81.5	1.3	0.7	1.6
HW		82								
MEAN		83.7	82.0	83.3						
S.D.		3.3	2.9	2.5						
S.E.		0.8	1.0	0.9						
% C.V.		4.0	3.6	3.0						

Total Population Variability

Mean = 82.6
S.D. = [illegible]
S.E. = [illegible]
% C.V. = [illegible]

Table A4 Nitroglycerin (GTN) plasma concentrations (ng/ml) after intravenous bolus doses of nitroglycerin injected into a femoral vein of sheep No. 13. (FA = femoral artery, FV = femoral vein)

	Dose of GTN (µg/kg)											
TIME	2.7		5.4		13.6		27.2		54.4		108.8	
(min)	FA	FV	FA	FV	FA	FV	FA	FV	FA	FV	FA	FV
0	15.2	0.23	38.4	3.4	72.3	3.5	145.6	1.9	304.8	302.4	496.3	211.0
1	0.82	[a]ND	3.5	2.2	7.0	7.4	27.2	16.8	81.6	36.8	172.1	46.0
2	0.24	ND	1.1	0.83	1.8	1.5	7.5	2.2	11.2	13.0	55.5	50.2
3	ND	ND	0.22	0.21	1.1	0.83	1.2	1.2	3.5	4.2	10.9	41.6
4	ND	ND	ND	ND	0.68	0.57	0.97	0.98	2.7	4.0	9.8	10.3
5	ND	ND	ND	ND	0.32	0.25	0.72	0.43	2.0	3.5	9.2	8.7
6	ND	ND	ND	ND	ND	ND	0.53	0.39	1.3	1.3	6.3	8.9
8	ND	ND	ND	ND	ND	ND	0.34	ND	0.50	0.63	3.5	3.8
10	ND	ND	ND	ND	ND	ND	0.20	ND	0.38	0.51	2.1	3.0
12	ND	ND	ND	ND	ND	ND	ND	ND	0.24	0.27	1.3	1.6
15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.72	1.2
$AUC_{0\rightarrow\infty}$	8.6	-	24.4	5.2	47.7	12.8	113.1	23.9	258.1	218.0	533.2	300.2
$AUMC_{0\rightarrow\infty}$	2.1	-	9.0	7.3	23.6	20.3	72.7	40.1	167.3	160.6	663.6	728.6
MRT	0.24	-	0.37	1.41	0.49	1.58	0.64	1.68	0.65	0.74	1.24	2.44

[a]not detectable

Table A5 Nitroglycerin (GTN) plasma concentrations (ng/ml) after intravenous bolus doses of nitroglycerin injected into a femoral vein of sheep No. 14. (FA = femoral artery, FV = femoral vein).

	Dose of GTN (µg/kg)											
Time	2.7		5.4		13.6		27.2		54.4		108.8	
(min)	FA	FV	FA	FV	FA	FV	FA	FV	FA	FV	FA	FV
0	9.8	0.33	38.0	2.1	45.2	2.9	97.3	3.7	311.0	110.0	420.0	410.8
1	1.1	[a]ND	3.8	1.1	9.2	4.7	15.3	10.5	78.2	114.0	151.8	130.1
2	0.38	ND	1.3	0.74	1.1	1.1	2.8	2.3	10.8	8.7	38.3	53.7
3	0.21	ND	0.43	[b]-	0.67	0.54	0.98	1.1	4.3	[b]-	10.0	12.1
4	ND	ND	0.25	ND	0.53	0.41	0.71	0.77	3.6	3.9	8.3	10.4
5	ND	ND	ND	ND	0.41	0.37	0.61	[b]-	2.9	2.7	7.8	7.8
6	ND	ND	ND	ND	0.27	0.24	0.41	[b]-	1.5	1.6	5.9	8.5
8	ND	ND	ND	ND	ND	ND	0.27	0.22	0.84	0.62	2.9	6.2
10	ND	ND	ND	ND	ND	ND	ND	ND	0.45	0.38	2.3	5.1
12	ND	ND	ND	ND	ND	ND	ND	ND	0.33	0.41	1.9	3.9
15	ND	ND	ND	ND	ND	ND	ND	ND	0.22	0.38	0.97	2.4
$AUC_{0\rightarrow\infty}$	6.6	-	25.1	4.0	35.6	9.4	70.8	18.6	262.0	198.3	457.5	485.3
$AUMC_{0\rightarrow\infty}$	2.4	-	11.3	7.9	25.4	19.0	45.6	37.9	211.0	263.5	682.4	1184.5
MRT	0.36	-	0.45	1.97	0.71	2.02	0.64	2.04	0.80	1.33	1.49	2.44

[a]not detectable
[b]sampling line blocked

Table A6 Nitroglycerin (GTN) plasma concentrations (ng/ml) after intravenous bolus doses of nitroglycerin injected into a femoral vein of sheep No. 15. (FA = femoral artery, FV = femoral vein).

	Dose of GTN (µg/kg)											
Time	2.7		5.4		13.6		27.2		54.4		108.8	
(min)	FA	FV	FA	FV	FA	FV	FA	FV	FA	FV	FA	FV
0	13.8	0.45	34.7	3.1	68.1	6.8	118.2	59.7	288.8	60.9	387.2	488.7
1	2.1	0.26	5.9	2.9	17.9	10.0	5.0	4.1	78.3	69.3	205.1	300.8
2	0.42	[a]ND	2.1	1.4	4.0	3.0	1.1	1.4	28.7	14.0	131.8	140.7
3	0.26	ND	1.4	0.68	2.2	1.6	0.73	0.99	8.3	7.3	46.3	121.7
4	ND	ND	0.94	0.44	1.9	1.1	0.67	0.84	6.1	7.0	18.9	50.8
5	ND	ND	0.59	0.23	1.2	0.81	0.53	0.58	3.7	3.1	7.4	28.7
6	ND	ND	ND	ND	0.64	0.57	0.29	0.31	2.4	2.1	6.0	12.7
8	ND	ND	ND	ND	0.47	0.24	ND	0.26	1.2	0.88	4.2	6.3
10	ND	ND	ND	ND	ND	ND	ND	ND	0.54	0.58	3.7	6.0
12	ND	ND	ND	ND	ND	ND	ND	ND	0.22	0.22	2.4	4.8
15	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.28	1.0	1.9
$AUC_{0\rightarrow\infty}$	9.9	-	29.5	7.6	64.0	21.7	68.0	39.3	277.4	138.4	639.7	957.0
$AUMC_{0\rightarrow\infty}$	3.9	-	31.1	14.9	70.1	43.6	22.9	29.6	266.6	236.1	1098.6	2022.0
MRT	0.39	-	1.05	1.96	1.09	2.01	0.34	0.75	0.96	1.71	1.71	2.11

[a]not detectable

Table A7 Reduction in mean arterial blood pressure (MABP) and mean left ventricular pressure (MLVP) induced by bolus doses of nitroglycerin (GTN) administered into a femoral vein of Sheep Nos. 13[a], 14[b] and 15[c].

Dose GTN (µg/kg)	% Reduction					
	a		b		c	
	MABP	MLVP	MABP	MLVP	MABP	MLVP
2.7	12.3	10.5	9.1	6.6	17.7	11.1
5.4	16.1	11.8	14.6	13.2	19.4	16.7
13.6	29.0	29.4	18.6	17.1	31.3	25.3
27.2	43.3	42.9	33.3	34.1	50.0	33.3
54.4	64.3	50.0	42.9	42.5	71.9	60.5
108.8	70.2	60.5	55.6	53.4	77.1	73.7

Table A8 The effect of bolus doses of nitroglycerin (GTN) on the duration of reduction in mean arterial blood pressure and their effects on cardiac output in sheep Nos. 13[a], 14[b], 15[c].

Dose GTN (µg/kg)	Duration of blood pressure reduction (min)			Maximum % change in cardiac output		
	a	b	c	a	b	c
2.7	1.4	1.2	1.3	+ 11.0	+ 6.1	+ 10.2
5.4	2.2	1.9	2.0	0	- 2.8	+ 6.3
13.6	3.0	3.6	4.2	- 2.0	- 4.1	0.3
27.2	8.0	8.4	9.4	- 2.7	- 4.8	- 1.2
54.4	20.0	24.0	22.0	- 6.5	- 8.0	- 4.8
108.8	28.0	29.1	25.5	- 17.5	- 20.1	- 13.9

Table A9 Nitroglycerin plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (0.4 µg/min/kg) into a femoral vein of sheep No. 9.

INFUSION	Time (min)	Portal vein	Hepatic vein	Pulmonary artery	Left ventricle	Femoral artery	Femoral vein
	10	1.4	[a]ND	6.7	4.7	4.2	0.42
	20	1.5	0.32	8.2	4.1	4.1	0.71
	30	1.5	0.34	7.9	4.7	4.6	0.63
	40	1.4	0.39	8.1	5.0	4.5	0.66
	41					1.6	0.23
	42					0.90	ND
	43					0.37	ND
	44					ND	ND
	45					ND	ND
	46					ND	ND
	48					ND	ND
	50					ND	ND
	52					ND	ND
	55					ND	ND
	60					ND	ND
	65					ND	ND
$AUC_{0\rightarrow\infty}$		59	10	316	189	156	21
$AUMC_{0\rightarrow\infty}$		1547	349	8485	5045	3820	570
MRT		26.22	32.34	26.85	26.63	24.42	26.52

[a]not detectable

Table A10 Nitroglycerin plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (0.4 µg/min/kg) into a femoral vein of sheep No. 16.

INFUSION	Time (min)	Portal vein	Hepatic vein	Pulmonary artery	Left ventricle	Femoral artery	Femoral vein
	10	1.0	[a]ND	5.5	2.5	2.2	ND
	20	1.2	ND	6.0	2.6	2.4	0.22
	30	1.2	ND	6.0	2.8	2.8	0.24
	40	1.2	0.26	5.8	2.8	2.7	0.28
	41					0.65	ND
	42					0.26	ND
	43					ND	ND
	44					ND	ND
	45					ND	ND
	46					ND	ND
	48					ND	ND
	50					ND	ND
	52					ND	ND
	55					ND	ND
	60					ND	ND
	65					ND	ND
$AUC_{0\rightarrow\infty}$		47	3	238	109	91	8
$AUMC_{0\rightarrow\infty}$		1263	125	6275	2926	2239	253
MRT		26.89	44.7	26.32	26.72	24.58	32.46

[a]not detectable

Table A11 Nitroglycerin plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (0.4 µg/min/kg) into a femoral vein of sheep No. 17.

INFUSION	Time (min)	Portal vein	Hepatic vein	Pulmonary artery	Left ventricle	Femoral artery	Femoral vein
	10	1.7	[a]ND	6.1	3.9	3.7	0.41
	20	1.8	0.28	6.2	3.9	3.8	0.52
	30	1.8	0.35	5.8	3.9	3.7	0.48
	40	1.8	0.42	6.0	3.9	3.8	0.64
	41					1.3	0.31
	42					0.78	0.20
	43					0.34	ND
	44					ND	ND
	45					ND	ND
	46					ND	ND
	48					ND	ND
	50					ND	ND
	52					ND	ND
	55					ND	ND
	60					ND	ND
	65					ND	ND
$AUC_{0\rightarrow\infty}$		72	11	246	159	137	19
$AUMC_{0\rightarrow\infty}$		1915	358	6410	4163	3267	502
MRT		26.39	32.54	26.02	26.10	23.74	26.42

[a]not detectable

Table A12 Nitroglycerin plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (5.7 µg/min/kg) into a femoral vein of sheep No. 10.

	Time (min)	Portal vein	Hepatic vein	Pulmonary artery	Left ventricle	Femoral artery	Femoral vein
INFUSION	10	10.3	3.4	92.7	67.1	65.2	12.3
	20	12.5	4.3	94.1	73.5	73.0	13.8
	30	11.7	3.8	105.3	70.2	68.9	13.3
	40	13.4	4.4	103.2	71.8	71.4	15.4
	41					40.8	8.8
	42					4.8	4.2
	43					2.2	2.0
	44					1.6	1.8
	45					1.2	1.2
	46					0.91	0.82
	48					0.63	0.60
	50					0.37	0.43
	52					0.22	0.20
	55					[a]ND	ND
	60					ND	ND
	65					ND	ND
$AUC_{0\rightarrow\infty}$		466	154	3849	2746	2517	505
$AUMC_{0\rightarrow\infty}$		12078	3994	98483	69296	59811	12485
MRT		25.92	25.84	25.58	25.22	23.76	24.72

[a]not detectable

Table A13 Nitroglycerin plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (5.7 µg/min/kg) into a femoral vein of sheep No. 11.

	Time (min)	Portal vein	Hepatic vein	Pulmonary artery	Left ventricle	Femoral artery	Femoral vein
INFUSION	10	13.4	4.3	115.3	79.1	76.0	10.0
	20	14.6	8.2	112.0	81.2	78.1	12.2
	30	22.4	8.5	122.4	82.2	85.2	11.0
	40	20.2	5.0	122.0	84.1	85.0	9.8
	41					62.0	6.7
	42					8.4	5.2
	43					3.7	3.2
	44					3.0	3.1
	45					2.1	2.0
	46					1.8	1.5
	48					1.2	1.2
	50					0.88	0.80
	52					0.64	0.71
	55					0.30	0.30
	60					ND	ND
	65					ND	ND
$AUC_{0\rightarrow\infty}$		717	262	4781	3298	2947	414
$AUMC_{0\rightarrow\infty}$		20132	6885	125806	86513	71276	10269
MRT		28.07	26.18	26.31	26.23	24.18	24.77

[a]not detectable

Table A14 Nitroglycerin plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (5.7 µg/min/kg) into a femoral vein of sheep No. 12.

	Time (min)	Portal vein	Hepatic vein	Pulmonary artery	Left ventricle	Femoral artery	Femoral vein
INFUSION	10	10.1	2.3	121.1	90.2	89.2	10.0
	20	14.3	4.7	123.8	92.9	92.8	13.4
	30	18.7	3.2	126.0	96.5	93.7	12.6
	40	18.1	4.8	127.3	99.2	97.1	12.1
	41					57.3	7.5
	42					8.9	5.4
	43					4.1	4.0
	44					2.9	3.4
	45					1.9	2.0
	46					1.6	1.4
	48					1.0	0.96
	50					0.77	0.77
	52					0.51	0.54
	55					0.26	ND
	60					[a]ND	ND
	65					ND	ND
$AUC_{0\rightarrow\infty}$		620	152	5051	3841	3371	545
$AUMC_{0\rightarrow\infty}$		17648	4454	132337	101476	80502	12372
MRT		28.46	29.30	26.20	26.42	23.88	22.7

[a]not detectable

Table A15 Nitroglycerin plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (22.1 µg/min/kg) into a femoral vein of sheep No. 18.

	Time (min)	Portal vein	Hepatic vein	Pulmonary artery	Left ventricle	Femoral artery	Femoral vein
INFUSION	10	50.8	34.6	252.7	161.2	143.2	34.5
	20	72.2	40.1	288.7	173.1	160.0	41.8
	30	83.1	55.4	283.8	170.5	163.7	37.5
	40	83.5	56.7	296.1	170.0	170.2	35.5
	41					68.7	24.8
	42					29.4	19.1
	43					17.4	16.8
	44					12.9	12.2
	46					9.0	8.5
	48					6.0	6.5
	50					3.8	3.9
	52					2.8	2.7
	55					1.6	1.5
	60					0.40	0.32
	65					[a]ND	ND
$AUC_{8\rightarrow\infty}$		2895	1867	11260	6740	5791	1464
$AUMC_{8\rightarrow\infty}$		79887	52185	294100	173950	141231	36846
MRT		27.59	27.95	26.12	25.80	24.39	25.17

[a]not detectable

Table A16 Nitroglycerin plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (22.1 µg/min/kg) into a femoral vein of sheep No. 19.

	Time (min)	Portal vein	Hepatic vein	Pulmonary artery	Left ventricle	Femoral artery	Femoral vein
INFUSION	10	43.2	18.1	258.6	133.0	131.5	42.3
	20	50.9	25.2	313.2	141.0	140.2	51.8
	30	57.6	27.3	360.5	168.0	166.2	57.6
	40	57.9	26.4	357.1	169.9	167.8	55.9
	41					83.2	33.9
	42					31.4	24.7
	43					20.0	19.3
	44					13.7	13.2
	46					8.3	8.0
	48					6.7	6.9
	50					4.0	4.2
	52					3.0	3.0
	55					1.7	1.6
	60					0.72	0.70
	65					0.44	0.40
$AUC_{8\rightarrow\infty}$		2110	974	12983	6165	5505	1982
$AUMC_{8\rightarrow\infty}$		57153	26559	352949	169545	137338	51384
MRT		27.09	27.27	27.83	27.50	24.95	25.92

Table A17 Nitroglycerin plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (22.1 µg/min/kg) into a femoral vein of sheep No. 20.

	Time (min	Portal vein	Hepatic vein	Pulmonary artery	Left ventricle	Femoral artery	Femoral vein
INFUSION	10	45.7	17.3	285.1	171.0	170.2	39.2
	20	50.3	15.5	321.8	173.2	171.8	40.8
	30	60.7	21.3	343.7	181.4	181.0	40.0
	40	61.8	22.1	341.9	181.0	180.2	41.5
	41					72.8	23.2
	42					25.9	18.7
	43					15.9	13.3
	44					12.3	10.8
	46					8.7	8.3
	48					6.0	6.0
	50					4.0	3.9
	52					2.9	3.0
	55					1.8	2.3
	60					0.79	0.70
	65					0.26	[a]ND
$AUC_{8\rightarrow\infty}$		2218	771	13120	7155	6405	1552
$AUMC_{8\rightarrow\infty}$		60718	21203	351055	188008	153813	38981
MRT		27.37	27.5	26.76	26.28	24.01	25.12

[a]not detectable

Table A18 Nitroglycerin plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (5.7 µg/min/kg) into a femoral vein of sheep No. 29. A bolus dose of glyceryl dinitrates (5mg) was injected into the aorta at the 30th minute of nitroglycerin infusion.

Time (min)	Portal vein	Hepatic vein	Pulmonary artery	Left ventricle	Femoral artery	Femoral vein
INFUSION 10	14.2	6.3	107.8	82.3	80.7	12.5
INFUSION 20	15.0	7.0	110.3	83.8	81.3	12.6
INFUSION 30	15.1	7.3	112.1	83.4	83.2	14.4
INFUSION 40	15.3	6.8	113.4	84.1	83.8	19.0
41					29.4	16.2
42					10.8	14.1
43					4.7	12.9
44					4.0	14.0
45					3.7	13.8
46					3.5	12.8
48					3.0	11.1
50					2.6	10.9
52					2.3	10.0
55					2.1	7.2
60					1.5	6.0
65					1.0	3.8
$AUC_{0\to\infty}$	598	285	4621	3470	3004	782
$AUMC_{0\to\infty}$	16120	7695	124280	92864	72002	28036
MRT	26.96	27.00	26.89	26.76	23.97	35.8

Table A19 Nitroglycerin plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (5.7 µg/min/kg) into a femoral vein of sheep No. 30. A bolus of glyceryl dinitrates (5mg) was injected into the aorta at the 30th minute of nitroglycerin infusion.

Time (min)	Portal vein	Hepatic vein	Pulmonary artery	Left ventricle	Femoral artery	Femoral vein
INFUSION 10	10.0	3.4	98.7	74.2	73.8	13.7
INFUSION 20	12.9	3.8	99.3	74.5	74.5	13.8
INFUSION 30	12.3	3.9	101.2	75.3	74.9	14.0
INFUSION 35	15.6	4.7	97.6	77.6	77.3	20.9
36					16.9	21.3
37					6.2	19.7
38					4.1	16.3
39					3.2	15.7
40					2.3	15.0
41					2.0	12.1
43					1.7	10.5
45					1.5	10.0
47					1.2	7.2
50					1.0	4.9
55					0.93	2.8
60					0.75	1.8
$AUC_{0\to\infty}$	439	157	3966	3016	2324	654
$AUMC_{0\to\infty}$	10465	4149	99070	75940	49655	18692
MRT	23.84	26.43	24.98	25.18	21.37	28.58

Table A20 Glyceryl dinitrate (1, 3-GDN, 1, 2-GDN) plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (0.4 µg/min/kg) into a femoral vein of sheep No. 16.

Time (min)	Portal vein		Hepatic vein		Pulmonary artery		Left ventricle		Femoral artery		Femoral vein	
	1,3-GDN	1,2-GDN	1,3-GDN	1,2-GDN	1,3-GDN	1,2-GDN	1,3-GDN	1,2-GDN	1,3-GDN	1,2-GDN	1,3-GDN	1,2-GDN
INFUSION 10	0.67	1.0	0.48	0.82	0.84	1.2	1.1	1.5	1.2	1.8	0.98	1.4
20	1.5	3.0	1.3	2.7	1.6	3.0	2.0	3.5	2.0	3.2	1.3	2.2
30	2.4	3.9	2.1	3.6	2.4	4.0	2.9	4.7	2.7	4.6	1.7	3.4
40	2.4	4.7	2.1	4.4	2.1	4.8	2.9	5.5	2.8	5.4	1.9	4.3
45									2.4	5.0	1.7	3.8
50	1.4	3.0	1.2	2.6	1.4	3.0	1.6	3.2	1.6	3.0	1.3	2.6
60									0.98	1.7	1.2	1.9
70	0.62	1.1	0.48	0.92	0.70	1.2	0.79	1.4	0.78	1.4	1.0	1.8
85									0.66	1.2	0.72	1.3
100									0.51	0.92	0.62	1.2
130									[a]ND	0.49	ND	0.55
$AUC_{0\rightarrow\infty}$	141	243	117	214	147	253	177	292	173	292	156	267
$AUMC_{0\rightarrow\infty}$	9794	14443	7863	12473	10575	15301	12274	17605	12049	17921	12877	18534
MRT	69.5	59.4	67.2	58.3	71.9	100.0	69.3	60.3	69.6	61.4	82.5	69.4

[a] not detectable

Table A21 Glyceryl dinitrate (1, 3-GDN, 1, 2-GDN) plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (0.4 µg/min/kg) into a femoral vein of sheep No. 17.

Time (min)	Portal vein		Hepatic vein		Pulmonary artery		Left ventricle		Femoral artery		Femoral vein	
	1,3-GDN	1,2-GDN	1,3-GDN	1,2-GDN	1,3-GDN	1,2-GDN	1,3-GDN	1,2-GDN	1,3-GDN	1,2-GDN	1,3-GDN	1,2-GDN
INFUSION 10	0.58	1.0	0.43	0.81	0.63	1.0	0.92	1.9	1.1	1.9	0.87	1.3
20	1.0	2.1	0.67	2.0	1.0	2.3	2.0	3.9	2.0	3.8	1.4	2.4
30	1.3	3.0	0.94	2.7	2.3	4.1	2.9	5.0	2.8	4.9	1.7	3.6
40	1.6	4.3	1.3	3.8	2.0	4.8	2.9	5.6	3.0	5.8	1.9	4.4
45									2.5	5.1	1.6	3.7
50	1.1	2.8	0.92	2.4	1.3	2.0	1.5	3.0	1.3	3.0	1.4	2.7
60									1.0	1.8	1.2	2.0
70	0.90	1.2	0.86	1.0	0.8	1.4	1.0	1.6	0.78	1.4	0.98	1.7
85									0.65	1.1	0.76	1.4
100									0.50	0.78	0.60	0.93
130									[a]ND	0.47	ND	0.55
$AUC_{0\rightarrow\infty}$	123	221	106	192	134	240	178	309	166	304	148	265
$AUMC_{0\rightarrow\infty}$	9563	13825	8788	11765	9350	15248	11925	18407	10457	17592	10993	17778
MRT	77.7	62.6	82.9	61.3	69.8	63.5	67.0	59.6	63.0	57.9	74.3	67.1

[a] not detectable

Table A22 Glyceryl mononitrate plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (0.4 µg/min/kg) into a femoral vein of sheep No. 16.

	Time (min)	Portal vein	Hepatic vein	Pulmonary artery	Left ventricle	Femoral artery	Femoral vein
INFUSION	10	0.52	0.47	0.60	0.87	0.83	0.41
	20	1.3	0.94	2.1	1.8	1.7	1.2
	30	1.6	1.2	3.4	3.1	3.0	1.5
	40	3.0	2.6	4.9	5.0	5.0	3.4
	45					6.0	4.3
	50	5.1	4.4	8.1	8.6	8.4	5.2
	60					9.4	5.7
	70	6.3	5.9	8.3	8.1	8.0	6.0
	85					8.2	6.5
	100					8.0	7.1
	130					7.4	7.9

Table A23 Glyceryl mononitrate plasma concentrations (ng/ml) after an intravenous infusion of nitroglycerin (0.4 µg/min/kg) into a femoral vein of sheep No. 17.

	Time (min)	Portal vein	Hepatic vein	Pulmonary artery	Left ventricle	Femoral artery	Femoral vein
INFUSION	10	0.63	0.47	1.2	1.2	1.0	0.43
	20	1.0	0.82	2.0	2.1	2.0	1.3
	30	2.3	2.0	3.5	4.3	4.3	2.8
	40	4.3	4.0	5.7	6.1	6.2	4.6
	45					7.4	5.0
	50	5.4	4.9	7.6	7.4	7.4	5.3
	60					7.0	6.3
	70	6.3	5.9	6.7	7.0	7.0	6.4
	85					6.9	7.4
	100					6.6	7.6
	130					6.2	7.6

Table A24 Aspirin (ASA) and salicylic acid (SA) plasma concentrations (µg/ml) after an intravenous infusion of aspirin (485 µg/min/kg) into a femoral vein of sheep No. 23.

Time	Portal Vein		Hepatic Vein		Pulmonary artery		Left ventricle		Femoral artery		Femoral vein	
(min)	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA
INFUSION 15	46.2	11.6	27.9	11.2	44.0	11.5	40.8	11.5	40.2	11.0	24.5	6.2
30	47.1	24.3	27.3	20.8	47.0	21.7	46.3	22.5	45.6	20.9	30.0	16.4
45	45.3	32.1	27.9	29.8	46.5	32.7	45.1	33.8	44.8	33.2	31.8	28.1
60	44.0	36.0	28.6	33.3	46.3	38.8	46.9	38.9	46.0	38.9	31.8	36.2
75	44.8	42.8	28.3	40.1	46.8	40.1	45.8	41.7	45.8	40.8	32.0	40.1
80									29.2	43.2	23.5	44.8
85									20.0	45.9	17.3	46.3
95	12.3	46.8	10.8	46.4	11.2	45.3	11.1	47.0	11.4	46.3	9.4	47.1
105									8.8	45.0	7.2	46.8
120									5.2	44.3	5.0	45.2
135									3.7	45.7	4.0	45.0
150									2.4	44.1	2.6	44.3
165									1.5	43.2	1.7	41.8
$AUC_{0\rightarrow\infty}$	4061	-	2652	-	4075	-	3982	-	3857	-	2768	-
$AUMC_{0\rightarrow\infty}$	226793	-	158782	-	226248	-	222885	-	216746	-	168653	-
MRT	55.8	-	59.9	-	55.5	-	56.0	-	56.2	-	60.9	-

Table A25 Aspirin (ASA) and salicylic acid (SA) plasma concentrations (µg/ml) after an intravenous infusion of aspirin (485 µg/min/kg) into a femoral vein of sheep No. 24.

Time	Portal Vein		Hepatic Vein		Pulmonary artery		Left ventricle		Femoral artery		Femoral vein	
(min)	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA
INFUSION 15	34.2	14.5	27.0	14.4	34.0	12.0	34.3	14.0	31.3	14.7	27.5	13.0
30	36.0	26.0	24.6	19.3	41.6	25.3	40.6	27.1	38.6	27.3	31.6	28.8
45	39.4	30.4	31.3	28.4	41.6	37.8	40.4	43.2	41.6	43.0	32.8	37.6
60	38.8	39.1	30.9	38.0	41.5	49.3	41.3	54.1	40.9	53.2	31.3	53.8
75	39.8	48.3	30.5	47.8	41.6	53.2	40.7	59.0	40.5	57.3	30.6	58.7
80									27.1	58.7	23.8	59.8
85									18.0	58.0	18.8	58.0
95									16.3	58.3	16.0	58.4
105	14.6	57.6	12.8	56.2	12.6	55.2	12.8	58.1	12.0	57.8	12.5	57.0
120									7.9	58.0	7.5	57.3
135									5.5	55.3	5.4	56.8
150									2.9	52.4	3.2	53.7
165									2.2	50.6	1.9	50.6
$AUC_{0\rightarrow\infty}$	3951	-	3042	-	3955	-	3915	-	3704	-	3134	-
$AUMC_{0\rightarrow\infty}$	249180	-	202037	-	245166	-	243662	-	223877	-	194697	-
MRT	63.1	-	66.4	-	62.0	-	62.2	-	60.4	-	62.1	-

Table A26 Aspirin (ASA) and salicylic acid (SA) plasma concentrations (µg/ml) after an intravenous infusion of aspirin (485 µg/min/kg) into a femoral vein of sheep No. 25.

Time	Portal Vein		Hepatic Vein		Pulmonary artery		Left ventricle		Femoral artery		Femoral vein	
(min)	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA
INFUSION 15	38.7	20.4	30.1	18.6	42.0	18.7	40.1	18.4	40.3	17.3	34.3	13.8
30	-[a]	-	32.4	32.3	44.1	35.6	44.1	34.6	43.1	34.8	38.7	30.5
45	-[a]	-	35.3	38.4	49.9	50.0	51.0	50.3	51.5	49.3	42.1	48.7
60	47.3	55.2	32.1	53.8	51.5	58.7	52.3	60.2	52.0	58.9	44.3	57.1
75	47.4	56.1	33.4	54.6	53.5	58.9	53.0	60.0	52.0	59.5	45.6	57.3
80									38.2	60.6	37.1	58.5
85									23.5	62.3	26.1	60.9
95									17.2	58.7	18.0	61.3
105									13.8	60.3	13.9	60.4
120									9.0	60.3	9.9	61.3
135									6.1	58.9	6.7	60.8
150									3.5	56.3	4.4	62.4
165									2.9	55.8	3.2	60.1
$AUC_{0\to\infty}$	4690	-	3422	-	5194	-	5170	-	4438	-	4040	-
$AUMC_{0\to\infty}$	325904	-	230956	-	361443	-	360032	-	275408	-	263634	-
MRT	69.5	-	67.5	-	69.6	-	69.6	-	62.1	-	65.2	-

[a] sampling line blocked

Table A27 Aspirin (ASA) and salicylic acid (SA) plasma concentrations (µg/ml) after an intravenous infusion of aspirin (485 µg/min/kg) into a femoral vein of sheep No. 28.

Time	Portal Vein		Hepatic vein		Pulmonary artery		Left ventricle		Femoral artery		Femoral vein	
(min)	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA
INFUSION 15	32.0	15.5	23.5	16.0	36.2	19.3	34.4	19.9	34.0	19.0	22.2	14.3
30	32.8	30.2	24.1	28.7	38.3	34.8	39.0	34.0	38.5	34.2	30.5	28.0
45	35.6	35.9	25.1	33.2	42.0	46.3	45.0	49.2	45.3	47.2	36.8	46.0
60	37.6	48.1	25.2	47.0	38.8	53.1	42.0	55.6	40.8	54.8	33.8	49.4
75	36.4	49.8	24.9	49.0	39.7	54.0	43.0	54.1	42.1	53.8	35.1	50.0
80									25.6	52.7	29.5	52.1
85									16.4	50.1	18.0	54.3
95	15.1	51.3	12.3	50.6	14.9	55.0	14.9	55.3	14.6	54.3	15.0	54.4
105									13.7	60.0	11.8	50.7
120	7.8	51.0	5.3	49.5	7.3	50.2	7.6	53.5	7.6	52.8	7.0	52.9
135									5.2	58.1	5.7	64.4
150									4.4	50.9	4.4	52.8
165									3.4	49.8	3.7	55.2
$AUC_{0\to\infty}$	3483	-	2456	-	3764	-	3906	-	3797	-	3225	-
$AUMC_{0\to\infty}$	227022	-	159380	-	235397	-	247358	-	245634	-	225476	-
MRT	65.2	-	64.9	-	62.5	-	63.3	-	64.7	-	69.9	-

Table A28 Total and free (fc) aspirin concentrations (µg/ml) and fraction unbound (fu) of aspirin in arterial and venous plasma of sheep No. 25 after a continuous intravenous infusion of aspirin (485 µg/min/kg).

		Arterial			Venous		
Time (min)		Total	fu	fc	Total	fu	fc
INFUSION	15	40.3	0.58	23.4	34.3	0.46	15.7
	30	43.1	0.62	26.7	38.7	0.52	20.1
	45	51.5	0.61	31.4	42.1	0.56	23.4
	60	52.0	0.66	34.3	44.3	0.56	24.7
	75	52.0	0.64	33.3	45.6	0.57	26.0
	80	38.2	0.71	27.1	37.1	0.62	22.9
	85	23.5	0.65	15.3	26.1	0.67	17.6
	95	17.2	0.73	12.5	18.0	0.63	11.3
	105	13.8	0.69	9.5	13.9	0.63	8.8
	120	9.0	0.70	6.3	9.9	0.71	7.0
	135	6.1	0.72	4.4	6.7	0.76	5.1
	150	3.5	0.74	2.6	4.4	0.70	3.1
	165	2.9	0.65	1.9	3.2	0.66	2.1
$AUC_{0\rightarrow\infty}$		4438		2845	4040		2306
$AUMC_{0\rightarrow\infty}$		275408		181288	263634		160323
MRT		62.1		63.7	65.2		69.5

Table A29 Total and free (fc) salicylic acid concentrations (µg/ml) and fraction unbound (fu) of salicylic acid in arterial and venous plasma of sheep No. 25 after a continuous intravenous infusion of aspirin (485 µg/min/kg).

		Arterial			Venous		
Time (min)		Total	fu	fc	Total	fu	fc
INFUSION	15	17.3	0.33	5.7	13.8	0.35	4.8
	30	34.8	0.28	9.7	30.5	0.30	9.1
	45	49.3	0.29	14.3	48.7	0.34	16.6
	60	58.9	0.33	19.4	57.1	0.29	16.6
	75	59.5	0.34	20.2	57.3	0.31	17.8
	80	60.6	0.35	21.3	58.5	0.36	21.1
	85	62.3	0.34	21.2	60.9	0.34	20.7
	95	58.7	0.38	22.5	61.3	0.32	19.6
	105	60.3	0.38	22.7	60.4	0.38	23.0
	120	60.3	0.35	20.9	61.3	0.35	21.4
	135	58.9	0.36	21.5	60.8	0.37	22.5
	150	56.3	0.36	20.3	62.4	0.37	23.1
	165	55.8	0.36	20.0	60.1	0.34	20.4

Table A30 Total and free (fc) aspirin concentrations (µg/ml) and fraction unbound (fu) of aspirin in arterial and venous plasma of sheep No. 28 after a continuous intravenous infusion of aspirin (485 µg/min/kg).

		Arterial			Venous		
Time (min)		Total	fu	fc	Total	fu	fc
INFUSION	15	34.0	0.66	22.4	22.2	0.75	16.7
	30	38.5	0.73	28.0	30.5	0.70	21.5
	45	45.3	0.76	34.3	36.8	0.65	24.0
	60	40.8	0.72	29.5	33.8	0.83	28.1
	75	42.1	0.74	31.0	35.1	0.78	27.5
	80	25.6	0.80	20.5	29.5	0.76	22.4
	85	16.4	0.78	12.9	18.0	0.79	14.3
	95	14.6	0.72	10.5	15.0	0.75	11.2
	105	13.7	0.67	9.2	11.8	0.78	9.2
	120	7.6	0.69	5.2	7.0	0.87	6.1
	135	6.2	0.73	3.8	5.7	0.71	4.0
	150	4.4	0.68	3.0	4.4	0.66	2.9
	165	3.4	0.70	2.4	3.7	0.70	2.6
$AUC_{0\rightarrow\infty}$		3797		2932	3225		2390
$AUMC_{0\rightarrow\infty}$		245634		174621	225476		165507
MRT		64.7		59.5	69.9		69.2

Table A31 Total and free (fc) concentrations of salicylic acid (µg/ml) and fraction unbound of salicylic acid in arterial and venous plasma of sheep No. 28 after a continuous intravenous infusion of aspirin (485 µg/min/kg).

		Arterial			Venous		
Time (min)		Total	fu	fc	Total	fu	fc
INFUSION	15	19.0	0.26	5.0	14.3	0.26	3.8
	30	34.2	0.26	8.8	28.0	0.26	7.3
	45	47.2	0.32	15.2	46.0	0.29	13.5
	60	54.8	0.29	15.8	49.4	0.33	16.4
	75	53.8	0.31	16.5	50.0	0.35	17.5
	80	52.7	0.31	16.5	52.1	0.35	18.0
	85	50.1	0.33	16.4	54.3	0.33	17.9
	95	54.3	0.31	16.7	54.4	0.33	18.1
	105	60.0	0.29	17.6	50.7	0.35	17.9
	120	52.8	0.31	16.2	52.9	0.36	18.8
	135	58.1	0.28	16.2	64.4	0.26	17.1
	150	50.9	0.31	15.8	52.8	0.30	16.1
	165	49.8	0.31	15.6	55.2	0.29	16.0

Table A32 Total and free salicyluric acid (SU) plasma concentrations (µg/ml) across a hind leg after an aspirin (ASA) infusion in sheep. (FA = femoral artery, FV = femoral vein, T = total, F = free).

		ASA 485 µg/min/kg Sheep No. 25				ASA 485 µg/min/kg Sheep No. 28				ASA 61 µg/min/kg + SA 1200 mg Sheep No. 29				ASA 61 µg/min/kg Sheep No. 30			
	Time (min)	FA T	FA F	FV T	FV F	FA T	FA F	FV T	FV F	FA T	FA F	FV T	FV F	FA T	FA F	FV T	FV F
INFUSION	15	2.1	[a]ND	1.8	ND	4.8	0.8	3.8	0.7	0.7	ND	0.6	ND	1.0	ND	0.9	ND
	30	4.6	1.1	4.0	1.0	7.3	2.2	6.0	2.0	2.2	ND	1.9	ND	1.4	ND	1.2	ND
	45	7.6	2.3	7.0	2.2	10.0	3.2	9.2	2.8	2.8	ND	2.6	ND	1.6	ND	1.4	ND
	60	9.3	3.2	7.7	2.7	10.0	3.0	8.6	3.2	7.3	2.8	4.7	2.0	2.1	ND	1.7	ND
	75	9.0	2.9	9.0	2.8	9.8	3.1	9.1	3.1	9.4	3.7	5.8	2.6	2.1	ND	2.2	ND
	80	8.7	3.0	9.1	3.3	9.9	3.1	9.9	3.3	10.2	3.9	7.7	3.6	2.0	ND	2.2	ND
	85	8.2	2.7	9.0	3.1	8.6	3.1	10.0	3.1	11.8	3.7	10.4	4.2	2.0	ND	2.1	ND
	95	10.6	3.4	9.8	3.1	10.2	3.1	10.1	3.4	13.2	4.2	11.5	4.0	1.7	ND	1.8	ND
	105	9.2	3.2	10.2	3.7	12.0	3.7	9.6	3.4	14.9	5.0	13.3	4.2	1.4	ND	1.7	ND
	120	11.0	3.7	10.3	3.3	11.5	3.5	11.5	4.0	14.0	5.1	15.3	5.2	1.6	ND	1.6	ND
	135	11.1	3.7	11.1	3.8	14.1	4.0	14.7	4.1	16.1	5.4	16.0	5.1	1.4	ND	1.5	ND
	150	12.3	3.8	13.6	3.8	13.9	4.4	13.5	4.0	17.1	5.8	16.2	5.4	1.1	ND	1.2	ND
	165	13.0	3.8	13.6	4.3	13.2	3.9	14.0	4.2	18.5	5.8	18.5	6.1	0.91	ND	1.0	ND

[a]not detected

Table A33 Time course of salicyluric acid (SU) plasma concentrations (µg/ml) across the liver after an aspirin infusion in sheep.

	ASA 485 µg/min/kg Sheep No. 25		ASA 485 µg/min/kg Sheep No. 28		ASA 61 µg/min/kg + SA 1200 mg Sheep No. 29		ASA 61 µg/min/kg Sheep No. 30	
Time (min)	Portal vein	Hepatic vein	Portal vein	Hepatic vein	Portal vein	Hepatic vein	Portal vein	Hepatic vein
INFUSION								
15	2.0	2.8	4.0	5.6	0.81	1.0	0.86	1.3
30	[a]-	6.3	5.3	7.0	2.0	2.3	1.4	2.3
45	-	8.3	6.8	8.2	2.8	3.6	1.9	2.7
60	8.3	9.8	7.8	9.4	7.4	11.1	2.1	3.5
75	8.3	9.9	8.8	10.2	8.8	12.4	2.6	4.0
85					10.2	13.8		
95			10.3	11.9			2.9	4.2
105			11.5	12.7	12.7	15.6	3.3	4.6

Table A34 Aspirin (ASA) and salicylic acid (SA) plasma concentrations (µg/ml) after an intravenous infusion of aspirin (61 µg/min/kg) into a femoral vein of sheep No. 30

Time	Portal Vein		Hepatic Vein		Pulmonary artery		Left ventricle		Femoral artery		Femoral vein	
(min)	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA
INFUSION 15	3.4	2.4	2.7	2.1	3.7	2.5	3.7	2.7	3.7	2.5	2.9	2.2
30	4.2	4.3	3.4	3.8	5.1	4.8	4.8	5.0	4.6	4.9	3.5	4.0
45	4.4	5.4	3.4	4.9	5.8	6.3	5.6	6.0	5.4	5.8	3.6	5.2
60	4.8	6.5	3.8	6.0	5.7	6.0	5.7	6.1	5.7	6.2	3.9	6.0
75	5.2	6.3	3.7	6.2	5.9	5.8	5.8	6.3	5.5	6.1	3.9	6.3
80									2.7	5.5	2.8	6.1
85									1.5	4.9	2.0	5.9
95	0.84	4.3	0.62	4.0	1.2	4.0	1.0	4.0	0.90	4.1	1.2	4.5
105									0.68	3.2	0.72	3.8
120									0.45	2.7	0.56	3.1
135									0.30	2.3	0.33	2.6
150									0.20	1.9	0.25	2.2
165									[a]ND	1.6	ND	1.8
$AUC_{0\to\infty}$	381	806	293	748	463	783	445	795	408	651	322	779
$AUMC_{0\to\infty}$	21645	102060	16329	88115	26924	86076	25355	87755	22613	81082	19262	88852
MRT	56.8	126.6	55.7	117.8	58.1	109.9	57.0	110.4	55.4	124.5	59.8	114.0

[a]not detectable

Table A35 Total and free (fc) aspirin concentrations (µg/ml) and fraction unbound (fu) of aspirin in arterial and venous plasma of sheep No. 30 after a continuous intravenous infusion of aspirin (61 µg/min/kg).

	Arterial			Venous		
Time (min)	Total	fu	fc	Total	fu	fc
INFUSION 15	3.7	0.38	1.4	2.9	0.40	1.1
30	4.6	0.43	2.0	3.5	0.48	1.7
45	5.4	0.44	2.4	3.6	0.52	1.9
60	5.7	0.44	2.5	3.9	0.51	2.0
75	5.5	0.45	2.5	3.9	0.51	2.0
80	2.7	0.44	1.2	2.8	0.48	1.3
85	1.5	0.38	0.57	2.0	0.44	0.88
95	0.90	0.41	0.37	1.2	0.44	0.53
105	0.68	0.35	0.24	0.72	0.41	0.30
120	0.45	-	[a]ND	0.56	0.46	0.24
135	0.30	-	ND	0.33	-	ND
150	0.20	-	ND	0.25	-	ND
165	ND	-	ND	ND	-	ND
$AUC_{0\to\infty}$	408		172	322		153
$AUMC_{0\to\infty}$	22613		9466	19262		9051
MRT	55.4		55.0	59.8		59.2

[a]not detectable

Table A36 Total and free (fc) salicylic acid concentrations (µg/ml) and fraction unbound (fu) of salicylic acid in arterial and venous plasma of sheep No. 30 after a continuous intravenous infusion of aspirin (61 µg/min/kg).

	Arterial			Venous		
Time (min)	Total	fu	fc	Total	fu	fc
INFUSION 15	2.5	0.27	0.73	2.2	0.24	0.51
30	4.9	0.25	1.2	4.0	0.25	1.0
45	5.8	0.24	1.4	5.2	0.26	1.3
60	6.2	0.26	1.6	6.0	0.26	1.6
75	6.1	0.28	1.7	6.3	0.30	1.9
80	5.5	0.27	1.5	6.1	0.26	1.6
85	4.9	0.26	1.3	5.9	0.25	1.5
95	4.1	0.24	1.0	4.5	0.26	1.2
105	3.2	0.22	0.70	3.8	0.27	1.0
120	2.7	0.24	0.65	3.1	0.23	0.71
135	2.3	0.26	0.60	2.6	0.24	0.62
150	1.9	0.25	0.47	2.2	0.25	0.55
165	1.6	0.25	0.40	1.8	0.27	0.49
$AUC_{0\to\infty}$	651		188	779		203
$AUMC_{0\to\infty}$	81082		20272	88852		23466
MRT	124.5		107.8	114.1		115.6

Table A37 Aspirin (ASA) and salicylic acid (SA) plasma concentrations (ug/ml) after an intravenous infusion of aspirin (61 ug/min/kg) into a femoral vein of sheep No. 27. A bolus dose of sodium salicylate equivalent to 30 mg salicylic acid was injected into the aorta at the 50th minute of aspirin infusion.

Time	Portal Vein		Hepatic Vein		Pulmonary artery		Left ventricle		Femoral artery		Femoral vein	
(min)	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA
INFUSION 15	4.8	2.4	4.0	2.6	6.0	2.1	5.8	2.7	5.2	2.7	2.8	1.8
INFUSION 30	7.3	3.8	5.0	3.7	8.5	5.0	8.2	5.4	8.2	5.2	5.0	4.0
INFUSION 45	8.0	6.0	6.8	5.8	[a]	[a]	8.9	6.1	8.8	6.1	5.2	7.1
INFUSION 60	8.0	15.8	6.9	14.4	8.8	12.8	8.6	16.0	8.3	15.2	4.9	18.1
INFUSION 75	7.9	13.1	6.7	12.8	8.2	10.9	8.2	12.2	8.2	12.0	5.0	15.2
80									5.3	11.3	4.4	14.6
85									3.3	10.4	4.0	14.4
95	2.5	8.3	1.9	7.1	2.4	8.0	2.4	8.2	2.4	8.0	2.4	11.7
105									1.8	7.2	1.8	9.8
120									1.2	6.2	1.1	8.4
135									0.82	4.9	1.0	6.5
150									0.57	4.0	0.80	5.0
165									0.40	3.3	0.51	3.9
$AUC_{0\rightarrow\infty}$	724	1333	546	1206	739	1241	729	1342	694	1352	483	1648
$AUMC_{0\rightarrow\infty}$	42811	146319	33839	128782	44262	137234	43521	144860	41875	148895	33754	185153
MRT	59.1	109.8	62.0	106.8	59.9	110.6	59.7	107.9	60.3	110.1	69.9	112.3

[a] sampling line blocked

Table A38 Total and free (fc) aspirin concentrations (ug/ml) and fraction unbound (fu) of aspirin in arterial and venous plasma of sheep No. 27 after a continuous intravenous infusion of aspirin (61 ug/min/kg) and a bolus dose of sodium salicylate equivalent to 30 mg salicylic acid.

	Arterial			Venous		
Time (min)	Total	fu	fc	Total	fu	fc
INFUSION 15	5.2	0.50	2.6	2.8	0.57	1.6
INFUSION 30	8.2	0.49	4.0	5.0	0.44	2.2
INFUSION 45	8.8	0.49	4.3	5.2	0.52	2.7
INFUSION 60	8.3	0.53	4.4	4.9	0.57	2.8
INFUSION 75	8.2	0.47	3.9	5.0	0.60	3.0
80	5.3	0.49	2.6	4.4	0.54	2.4
85	3.3	0.51	1.7	4.0	0.50	2.0
95	2.4	0.40	0.95	2.4	0.48	1.1
105	1.8	0.32	0.57	1.8	0.40	0.72
120	1.2	0.35	0.42	1.1	0.45	0.49
135	0.82	0.36	0.30	1.0	0.34	0.34
150	0.57	0.38	0.22	0.8	0.35	0.28
165	0.40	-	[a] ND	0.51	-	[a] ND
$AUC_{0\rightarrow\infty}$	694	331		483	241	
$AUMC_{0\rightarrow\infty}$	41875	19082		33754	15992	
MRT	60.3	57.6		69.9	66.3	

[a] not detectable

Table A39 Total and free (fc) salicylic acid concentrations (ug/ml) and fraction unbound (fu) of salicylic acid in arterial and venous plasma of sheep No. 27 after a continous intravenous infusion of aspirin (61 ug/min/kg) and a bolus dose of sodium salicylate equivalent to 30 mg salicylic acid.

	Arterial			Venous		
Time (min)	Total	fu	fc	Total	fu	fc
INFUSION 15	2.7	0.30	0.82	1.8	0.37	0.67
INFUSION 30	5.2	0.27	1.4	4.0	0.30	1.2
INFUSION 45	6.1	0.34	2.1	7.1	0.27	1.9
INFUSION 60	15.2	0.25	3.8	18.1	0.24	4.4
INFUSION 75	12.0	0.22	2.6	15.2	0.26	4.0
80	11.3	0.22	2.4	14.6	0.26	3.8
85	10.4	0.22	2.2	14.4	0.25	3.6
95	8.0	0.25	2.0	11.7	0.24	2.8
105	7.2	0.24	1.7	9.8	0.23	2.2
120	6.2	0.26	1.6	8.4	0.25	2.1
135	4.9	0.25	1.2	6.5	0.27	1.8
150	4.0	0.24	0.96	5.0	0.26	1.3
165	3.3	0.24	0.8	3.9	0.28	1.1
$AUC_{0\rightarrow\infty}$	1352	333		1648	430	
$AUMC_{0\rightarrow\infty}$	148895	36321		185153	48935	
MRT	110.1	109.1		112.3	113.8	

Table A40 Aspirin (ASA) and salicylic acid (SA) plasma concentrations (µg/ml) after an intravenous infusion of aspirin (61 µg/min/kg) into a femoral vein of sheep No. 26. A bolus dose of sodium salicylate equivalent to 300 mg salicylic acid was injected into the aorta at the 50th minute of aspirin infusion.

Time (min)	Portal Vein		Hepatic Vein		Pulmonary artery		Left ventricle		Femoral artery		Femoral vein	
	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA
INFUSION 15	4.4	1.3	3.7	1.5	5.3	1.0	4.8	1.8	4.9	1.6	3.4	1.6
INFUSION 30	5.9	3.7	4.3	3.4	5.7	3.2	5.6	2.4	5.6	2.4	4.8	3.2
INFUSION 45	5.3	5.1	4.7	4.9	7.0	5.7	7.2	5.0	7.0	4.8	6.0	5.0
INFUSION 60	7.4	68.8	6.3	54.4	7.7	53.4	7.6	58.7	7.5	57.6	6.6	63.2
INFUSION 75	7.4	51.3	6.0	49.8	7.8	32.9	7.8	36.1	7.6	36.0	6.5	55.3
80									5.5	32.5	6.0	38.0
85									4.2	31.0	4.5	34.0
95	2.9	26.2	1.8	24.1	3.1	22.7	3.1	23.8	3.0	23.0	3.0	28.5
105									2.0	21.2	2.1	25.3
120									1.4	17.9	1.6	20.0
135									1.0	14.0	1.1	16.5
150									0.71	12.2	0.77	13.3
165									0.44	9.8	0.44	10.6
$AUC_{0\rightarrow\infty}$	619	4137	480	3726	677	3307	669	3518	632	3652	580	4246
$AUMC_{0\rightarrow\infty}$	42010	452932	30915	412268	45335	372517	45222	394197	41445	422832	39723	482663
MRT	67.8	109.5	64.4	110.6	67.0	112.6	67.6	112.0	65.6	115.8	68.5	113.7

Table A41 Total and free (fc) aspirin concentrations (ug/ml) and fraction unbound (fu) of aspirin in arterial and venous plasma of sheep No. 26 after a continuous intravenous infusion of aspirin (61 ug/min/kg) and a bolus dose of sodium salicylate equivalent to 300 mg salicylic acid.

Time (min)	Arterial			Venous		
	Total	fu	fc	Total	fu	fc
INFUSION 15	4.9	0.47	2.3	3.4	0.47	1.6
INFUSION 30	5.6	0.48	2.7	4.8	0.48	2.3
INFUSION 45	7.0	0.48	3.4	6.0	0.45	2.7
INFUSION 60	7.5	0.49	3.7	6.6	0.51	3.3
INFUSION 75	7.6	0.50	3.8	6.5	0.52	3.4
80	5.5	0.46	2.5	6.0	0.50	3.0
85	4.2	0.47	1.4	4.5	0.46	2.1
95	3.0	0.44	0.95	3.0	0.48	1.4
105	2.0	0.48	0.65	2.1	0.40	0.85
120	1.45	0.47	0.50	1.6	0.44	0.73
135	1.0	0.48	0.38	1.1	0.45	0.50
150	0.71	0.42	0.30	0.77	0.50	0.38
165	0.44	-	[a]ND	0.44	-	ND
$AUC_{0\rightarrow\infty}$	632	287		580	274	
$AUMC_{0\rightarrow\infty}$	41445	18142		39723	19363	
MRT	65.6	63.2		68.5	70.7	

[a]not detectable

Table A42 Total and free (fc) salicylic acid concentrations (µg/ml) and fraction unbound (fu) of salicylic acid in arterial and venous plasma of sheep No. 26 after a continuous intravenous infusion of aspirin (61 µg/min/kg) and a bolus dose of sodium salicylate equivalent to 300 mg salicylic acid.

	Arterial			Venous		
	Total	fu	fc	Total	fu	fc
INFUSION 15	1.6	0.30	0.48	1.6	0.29	0.46
INFUSION 30	2.4	0.33	0.80	3.2	0.34	1.1
INFUSION 45	4.8	0.29	1.4	5.0	0.27	1.3
INFUSION 60	57.6	0.23	13.5	63.2	0.32	20.2
INFUSION 75	36.0	0.30	12.0	55.3	0.31	17.3
80	32.5	0.28	9.0	38.0	0.35	13.3
85	31.0	0.23	7.1	34.0	0.31	10.4
95	23.0	0.27	6.1	28.5	0.29	8.2
105	21.2	0.25	5.3	25.3	0.30	7.6
120	17.9	0.24	4.3	20.0	0.28	5.5
135	14.0	0.26	3.7	16.5	0.30	4.9
150	12.2	0.21	2.6	13.3	0.26	3.5
165	9.8	0.20	2.0	10.6	0.26	2.8
$AUC_{0\rightarrow\infty}$	3652	899		4246	1261	
$AUMC_{0\rightarrow\infty}$	422832	98984		482663	138323	
MRT	115.8	110.1		113.7	109.7	

Table A43 Aspirin (ASA) and salicylic acid (SA) plasma concentrations (ug/ml) after an intravenous infusion of aspirin (61 µg/min/kg) into a femoral vein of sheep No. 29. A bolus dose of sodium salicylate equivalent to 1200 mg salicylic acid was injected into the aorta at the 50th minute of aspirin infusion.

Time (min)	Portal vein		Hepatic vein		Pulmonary artery		Left ventricle		Femoral artery		Femoral vein	
	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA	ASA	SA
INFUSION 15	3.5	2.0	3.0	1.8	3.5	1.2	3.4	1.2	3.3	1.1	2.6	0.81
INFUSION 30	3.9	2.7	3.0	2.5	4.0	2.8	3.8	2.7	3.9	2.5	3.0	2.3
INFUSION 45	4.3	3.8	3.3	3.7	4.1	4.6	4.1	4.0	4.1	3.8	3.3	3.9
INFUSION 60	4.8	232.7	4.0	208.3	4.0	210.1	4.3	214.3	4.0	212.1	2.8	240.8
INFUSION 75	3.4	164.0	2.7	151.8	3.7	155.9	3.0	164.1	2.8	163.2	2.2	152.3
80									1.8	155.3	1.8	155.7
85	1.0	153.7	0.72	150.1	1.2	146.3	1.2	149.0	1.2	148.0	1.5	157.6
95									0.69	144.7	0.82	155.3
105	0.56	136.3	0.42	134.1	0.46	138.6	0.48	140.0	0.44	138.5	0.50	141.3
120									[a]ND	120.8	0.29	132.0
135									ND	111.6	ND	123.7
150									ND	107.5	ND	119.2
165									-	-	-	-
$AUC_{0\to\infty}$	320	-	256	-	341	-	303	-	293	-	240	-
$AUMC_{0\to\infty}$	15112	-	12704	-	17288	-	15057	-	14332	-	12448	-
MRT	47.2	-	49.6	-	50.7	-	49.7	-	48.9	-	51.9	-

[a]not detected

Table A44 Total and free (fc) aspirin concentrations (µg/ml) and fraction unbound (fu) of aspirin in arterial and venous plasma of sheep No. 29 after a continuous intravenous infusion of aspirin (61 µg/min/kg) and a bolus dose of sodium salicylate equivalent to 1200 mg salicylic acid.

	Arterial			Venous		
Time (min)	Total	fu	fc	Total	fu	fc
INFUSION 15	3.3	0.51	1.7	2.6	0.46	1.2
INFUSION 30	3.9	0.49	1.9	3.0	0.50	1.5
INFUSION 45	4.1	0.51	2.1	3.3	0.54	1.8
INFUSION 60	4.0	0.88	3.5	2.8	0.96	2.7
INFUSION 75	2.8	1.0	2.8	2.2	1.0	2.2
80	1.8	1.0	1.8	1.8	1.0	1.8
85	1.2	1.0	1.2	1.5	1.0	1.5
95	0.69	0.99	0.70	0.82	0.99	0.84
105	0.44	1.0	0.43	0.50	1.0	0.49
120	[a]ND	-	ND	0.29	1.0	0.30
135	-	-	-	ND	-	ND
150	-	-	-	-	-	-
165	-	-	-	-	-	-
$AUC_{0\to\infty}$	293		201	240		173
$AUMC_{0\to\infty}$	14332		11264	12448		10397
MRT	48.9		56.0	51.9		60.1

[a]not detectable

Table A45 Total and free (fc) salicylic acid concentrations (ug/ml) and fraction unbound (fu) of salicylic acid in arterial and venous plasma of sheep No. 29 after a continuous intravenous infusion of aspirin (61 µg/min/kg) and a bolus dose of sodium salicylate equivalent to 1200 mg salicylic acid.

	Arterial			Venous		
Time (min)	Total	fu	fc	Total	fu	fc
INFUSION 15	1.1	0.27	0.30	0.81	0.25	0.20
INFUSION 30	2.5	0.26	0.60	2.3	0.28	0.61
INFUSION 45	3.8	0.26	1.0	3.9	0.25	1.0
INFUSION 60	212.1	0.38	80.0	240.8	0.46	112.4
INFUSION 75	163.2	0.37	60.9	152.3	0.44	67.0
80	155.3	0.36	55.9	155.7	0.45	70.0
85	148.0	0.37	55.2	157.6	0.47	74.3
95	144.7	0.37	53.5	155.3	0.43	66.8
105	138.5	0.35	48.4	141.3	0.40	56.0
120	120.8	0.36	43.8	132.0	0.40	52.9
135	111.6	0.36	40.2	123.7	0.42	52.0
150	107.5	0.37	39.8	119.2	0.42	50.1
165	-	-	-	-	-	-

Table A46 Platelet aggregation, MDA synthesis and plasma aspirin concentrations on the seventh day after Astrix 20 mg, 1 capsule daily for seven days.

Subject	Platelet aggregation % Inhibition of control: Adrenalin T	Adrenalin P	Collagen T	Collagen P	Arachidonic acid T	Arachidonic acid P	MDA synthesis % Inhibition of control: Arachidonic acid T	Arachidonic acid P	NEM T	NEM P	Plasma aspirin concentration (ug/ml) T	Plasma aspirin concentration (ug/ml) P
KS	-	-	0	9	-	97	-	46	38	30	< 0.10	< 0.10
EM	0	0	0	0	-	-	-	-	47	-	< 0.10	< 0.10
DK	-	-	0	0	-	97	-	41	56	40	< 0.10	< 0.10
SS	12	10	13	13	-	-	-	-	36	39	< 0.10	< 0.10
DR	23	12	16	4	-	-	-	-	32	11	< 0.10	< 0.10
HE	14	28	14	7	-	-	-	-	20	3	< 0.10	< 0.10
SG	13	10	9	0	-	100	-	51	63	32	< 0.10	< 0.10
MW	0	7	2	12	-	-	-	-	40	6	< 0.10	< 0.10
MEAN	10.3	11.2	6.7	5.6		98.0		46.0	41.5	23.0	< 0.10	< 0.10
S.D.	8.9	9.3	7.0	5.4		1.7		5.0	13.6	15.8		
S.E.	3.6	3.8	2.5	1.9		1.0		3.0	4.8	6.0		

* T = Trough sample: 1 hr before final dose
P = Peak sample : 3 hrs after final dose

Table A47 Platelet aggregation, MDA synthesis and plasma aspirin concentrations on the seventh day after Astrix 50 mg, 1 capsule daily for seven days.

Subject	Platelet aggregation % Inhibition of control: Adrenalin T	Adrenalin P	Collagen T	Collagen P	Arachidonic acid T	Arachidonic acid P	MDA synthesis % Inhibition of control: Arachidonic acid T	Arachidonic acid P	NEM T	NEM P	Plasma aspirin concentration (ug/ml) T	Plasma aspirin concentration (ug/ml) P
RH	80	82	0	0	-	-	-	-	68	72	< 0.10	< 0.10
AU	69	69	16	16	-	-	-	-	85	85	< 0.10	< 0.10
MS	62	56	10	10	-	98	-	61	32	77	< 0.10	0.10
SJ	-	-	37	-	-	-	-	-	48	-	< 0.10	< 0.10
WM	71	77	0	21	-	100	-	53	29	97	< 0.10	< 0.10
KN	21	54	14	14	-	-	-	-	53	100	< 0.10	< 0.10
JC	78	83	8	8	-	96	-	56	19	98	< 0.10	0.10
HW	-	-	34	34	-	-	-	-	77	99	< 0.10	< 0.10
MEAN	63.5	70.2	14.9	14.7		98.0	-	56.7	51.4	89.6	< 0.10	< 0.10
S.D.	21.8	12.8	14.0	10.8		2.0	-	4.0	23.9	11.7		
S.E.	8.9	5.2	5.0	4.1		1.1	-	2.3	8.4	4.4		

*T = Trough sample: 1 hr before final dose
P = Peak sample : 3 hrs after final dose

Table A48 Platelet aggregation, MDA synthesis and plasma aspirin concentrations on the seventh day after Astrix 100 mg, 1 capsule daily for seven days.

Subject	Platelet aggregation % Inhibition of control: Adrenalin T	Adrenalin P	Collagen T	Collagen P	Arachidonic acid T	Arachidonic acid P	MDA synthesis % Inhibition of control: Arachidonic acid T	Arachidonic acid P	NEM T	NEM P	Plasma aspirin Concentration (ug/ml) T	Plasma aspirin Concentration (ug/ml) P
KS	-	-	53	53	-	100	-	93	82	83	< 0.10	< 0.10
EM	59	62	15	28	-	-	-	-	80	89	< 0.10	0.15
DK	75	56	52	50	-	98	-	52	73	84	< 0.10	< 0.10
SS	62	68	39	32	-	-	-	-	90	97	< 0.10	0.60
DR	39	47	8	31	-	99	-	88	63	77	< 0.10	< 0.10
HE	59	62	0	16	-	-	-	-	82	100	< 0.10	0.20
SG	2	26	4	0	-	100	-	82	70	100	< 0.10	< 0.10
MW	-	62	-	17	-	-	-	-	87	99	< 0.10	0.25
MEAN	49.3	54.7	24.4	28.4		99.2		78.8	78.4	91.1	< 0.10	0.15
S.D.	25.9	14.3	22.9	17.6		1.0		18.6	9.0	9.1		0.21
S.E.	10.6	5.4	8.6	6.3		0.5		9.3	3.2	3.2		0.07

*T = Trough sample: 1 hr before final dose
P = Peak sample : 3 hrs after final dose

Table A49 Platelet aggregation, MDA synthesis and plasma aspirin concentrations on the seventh day after Astrix 200 mg, 1 capsule daily for seven days.

Subject	Platelet aggregation % Inhibition of control: Adrenalin T	Adrenalin P	Collagen T	Collagen P	Arachidonic acid T	Arachidonic acid P	MDA Synthesis % Inhibition of control: Arachidonic acid T	Arachidonic acid P	NEM T	NEM P	Plasma aspirin concentration (ug/ml) T	Plasma aspirin concentration (ug/ml) P
RH	67	69	34	37	-	100	-	93	-	87	< 0.10	0.30
AU	71	77	33	33	-	-	-	-	83	90	< 0.10	< 0.10
MS	-	-	-	-	-	100	-	89	-	90	< 0.10	-
SJ	86	92	34	60	-	-	-	-	87	93	< 0.10	0.20
WM	77	79	32	31	-	99	-	88	89	96	< 0.10	0.25
KN	74	69	28	30	-	-	-	-	-	92	< 0.10	0.30
JC	88	82	46	55	-	98	-	68	70	78	< 0.10	< 0.10
HW	-	-	56	58	-	-	-	-	88	89	< 0.10	0.35
MEAN	77.2	78.0	37.6	43.4		99.2		84.7	83.4	89.4	< 0.10	0.21
S.D.	8.3	8.7	9.8	13.6		1.0		11.1	7.8	5.3		0.15
S.E.	3.4	3.5	3.7	5.1		0.5		5.5	3.2	1.2		0.06

*T = Trough sample: 1 hr before final dose
P = Peak sample : 3 hrs after final dose

Table A50 Platelet aggregation, MDA synthesis and plasma aspirin concentrations on the seventh day after Astrix 650 mg, 1 capsule daily for seven days.

Subject	Platelet aggregation % Inhibition of control: Adrenalin T	Adrenalin P	Collagen T	Collagen P	Arachidonic acid T	Arachidonic acid P	MDA synthesis % Inhibition of control: Arachidonic acid T	Arachidonic acid P	NEM T	NEM P	Plasma aspirin concentration (ug/ml) T	Plasma aspirin concentration (ug/ml) P
KS	-	-	56	64	96	100	97	97	84	99	< 0.10	0.25
EM	45	38	23	24	97	100	92	98	92	100	< 0.10	0.15
DK	-	-	64	70	100	100	97	97	90	97	< 0.10	0.10
SS	52	67	40	48	100	100	96	96	89	100	< 0.10	2.20
DR	67	73	19	31	89	93	90	76	90	95	< 0.10	0.65
HE	-	-	19	52	90	94	91	79	79	94	< 0.10	0.25
SG	49	49	11	19	86	85	99	96	82	97	< 0.10	1.35
MW	-	-	0	6	86	98	96	95	83	96	< 0.10	0.50
MEAN	53.2	56.7	29.0	39.2	93.0	96.2	94.7	91.9	86.1	97.2	< 0.10	0.65
S.D.	9.6	16.1	22.3	22.7	5.9	5.4	3.3	8.9	4.7	2.2		0.73
S.E.	4.8	8.0	7.9	8.1	2.1	1.9	1.2	3.1	1.7	0.8		0.26

*T = Trough sample: 1 hr before final dose
P = Peak sample : 3 hrs after final dose

Table A51 Platelet aggregation, MDA synthesis and plasma aspirin concentrations on the seventh day after Astrix 1300 mg, 1 capsule daily for seven days.

Subject	Platelet aggregation % Inhibition of control: Adrenalin T	Adrenalin P	Collagen T	Collagen P	Arachidonic acid T	Arachidonic acid P	MDA Synthesis % Inhibition of control: Arachidonic acid T	Arachidonic acid P	NEM T	NEM P	Plasma aspirin concentration (ug/ml) T	Plasma aspirin concentration (ug/ml) P
RH	-	-	34	43	92	99	97	97	90	99	< 0.10	3.0
AU	76	64	24	21	95	100	88	93	81	88	< 0.10	0.20
MS	55	62	19	26	99	100	83	99	94	99	< 0.10	0.95
SJ	-	-	21	65	97	100	94	98	92	96	< 0.10	1.0
WM	69	77	45	69	100	100	81	91	84	97	< 0.10	1.35
KN	63	64	23	24	97	100	71	-	-	-	< 0.10	0.15
JC	-	-	67	64	100	100	91	73	86	93	< 0.10	1.0
HW	-	-	67	71	100	100	82	-	-	-	< 0.10	4.25
MEAN	65.7	66.7	37.5	47.9	97.5	99.9	85.9	92.0	87.8	95.0	< 0.10	1.31
S.D.	8.9	6.9	20.0	21.8	2.9	0.3	8.3	9.7	5.0	4.2		1.42
S.E.	4.4	3.4	7.1	7.8	1.0	0.1	2.9	4.0	2.0	1.7		0.76

*T = Trough sample: 1 hr before final dose
P = Peak sample : 3 hrs after final dose

Table A52 Effects of nitroglycerin infusions on the cardiac output and mean arterial blood pressure of sheep just before termination of the infusion.

Rate of Infusion µg/min/kg	% change in cardiac output	% reduction in mean arterial blood pressure
0.4	+ 7.1	0
	+ 1.6	2.6
	- 5.4	1.6
5.7	+ 2.7	14.5
	+ 5.3	9.1
	+ 7.6	3.6
22.1	+ 3.8	16.7
	+ 17.0	6.4
	+ 11.2	18.8

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